

**Effects of heat shock, hypoxia, post-mortem
interval and glioma disease state on heat shock
gene *HSPA* expression**

by

Glenda Maria Beaman

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Student Declaration

I declare that while registered as a candidate for the research degree, I have not been a registered candidate or enrolled student for another award of the University or other academic or professional institution.

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Abstract

Heat shock protein 70 (*HSPA/HSP70*) gene expression is induced by a wide range of cellular stress conditions. This study investigated *HSPA/HSP70* expression in human cell lines exposed to hypoxic conditions, in cancerous and non-cancerous brain tissue specimens from 18 patients (gliomas and normal conditions), and in post mortem rat brain samples exposed to heat shock.

Three human glioma cell lines were chosen for this study, each representing various types of glioma: (astrocytoma, oligodendroglioma and glioblastoma), with a normal human astrocyte cell line used as a control. In addition, 18 clinical brain tissue samples were also examined. *HSPA* RNA transcripts and proteins were examined in these samples using qRT-PCR, immunofluorescence and flow cytometry techniques.

The average *HSPA* mRNA copy numbers detected in glioblastoma tissue were 1.8 and 8.8 fold higher respectively than in lower grade glioma and control tissues, which is suggestive of a grade related transcription profile. Similar patterns of grade related expression were also observed in corresponding cell lines. The percentage of cells showing positive for *HSPA* protein in normal cell lines increased from 0 to 33% immediately after exposure to hypoxia, and gradually declined to 11% 24 h after treatment. However, the effects of hypoxia were marginal in glioma cells, due to the already elevated levels of *HSPA*. Although hypoxia induced *HSPA* expression in normal cells, it did not achieve the same level of induction in cancer cells, suggesting that there are other factors which contribute to the induction of *HSPA*. These results suggest that *HSPA* is induced in cancer cells, not only by hypoxia, but also by other factors. In addition, this study indicated for the first time that *HSPA* expression in

glioma cells may possibly be grade related, and thus may have value as a prognostic marker. However a greater sample size is needed to validate such findings.

This study showed that *HSPA* is expressed at low levels in normal brain tissue, but was more highly expressed in brain tissue subjected to mild heat shock. The levels of *HSPA* transcripts in heat shocked post mortem brain tissue showed a marked increase in *HSPA* expression.

GAPDH was used as a control gene for these studies, and exhibited a consistent level of expression in normal and tumourous cell lines and tissue samples under normal and hypoxic conditions, and also in post mortem tissues exposed to heat shock. For *Homo sapiens GAPDH*, the average transcript numbers for normal and tumourous cell lines and brain tissue samples were approximately 145,000 copies per sample. For *Rattus norvegicus GAPDH*, levels were higher than for human samples, at an average of 268,300 copies per sample. The consistency of these results confirms that *GAPDH* was a suitable candidate gene for the purpose of this study.

Early in the post-mortem period, *HSPA* is expressed more highly in tissues subjected to single and multiple heat shocks compared to controls. However, later post-mortem intervals of between 3 - 24 h demonstrated inconsistent and irregular results, with no predictive or reproducible patterns. Therefore, although there is demonstrable *de novo* expression of *HSPA* in post mortem brain tissue in response to heat shock, it is difficult to predict the full parameters of this induction, probably as a result of other forms of cellular stress affecting these tissues under our experimental methodology. These initial studies indicate that the use of *HSPA* with the methodologies employed here are not suitable as an accurate indicator of post-mortem interval.

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Abbreviations

| | |
|---------|--|
| μl | Microlitres |
| μg | Micrograms |
| 1321N1 | Grade I astrocytoma cell line |
| A | Adenosine |
| A172 | Adult malignant glioma cell line |
| aa-tRNA | Amino acid transfer ribonucleic acid |
| ABM | Astrocyte Basal Medium |
| AKT1 | RAC-alpha serine/threonine protein kinase |
| AM | Astrocyte medium |
| AMV | Avian Myeloblastosis Virus |
| ADP | Adenosine diphosphate |
| ARNT | Aryl hydrocarbon receptor nuclear translocator |
| ATCC | American Type Culture Collection |
| ATP | Adenosine triphosphate |
| BAG-1 | Bcl-2-associated athanogene-1 |
| bHLH | Basic helix loop helix |
| bp | Base Pairs |
| BSA | Bovine Serum Albumen |
| C | Cytosine |
| c-CND1 | Condensin, non-SMC subunit |
| c-FOS | FBJ osteosarcoma oncogene |
| c-MYC | Myelocytomatosis oncogene |

| | |
|--------|--|
| cDNA | Complimentary DNA |
| CHIP | Carboxy-terminus OH Hsp70 Interacting Protein |
| COOH | Carboxylic acid |
| CNS | Central Nervous System |
| Ct | Cycle threshold |
| CT | Computed tomography |
| CYR61 | Cysteine-rich, angiogenic inducer, 61 |
| D341MG | Pediatric medulloblastoma cell line |
| D392MG | Adult malignant glioma cell line |
| D54MG | Adult malignant glioma cell line |
| dATP | Deoxyadenosine triphosphate |
| DAXX7 | Cellular death associated protein |
| DBD | DNA binding domain |
| dCTP | Deoxycytidine triphosphate |
| DDBJ | DNA Data Bank of Japan |
| dGTP | Deoxyguanosine triphosphate |
| DMEM | Dulbeccos Modified Eagles Medium |
| DMSO | Dimethylsulphoxide |
| DMSZ | Deutsche Sammlung von Mikroorganismen und Zellkulturen |
| DNA | Deoxyribonucleic Acid |
| DNAJ | Heat shock protein 40 |
| dsDNAs | Double Stranded DNA |
| dTTP | Deoxythymidine triphosphate |

| | |
|---------|---|
| DTT | Dithiothreitol |
| EBI | European Bioinformatics Institute |
| ECACC | European Collection of Cell Cultures |
| EDTA | Ethylenediaminetetraacetic Acid |
| EMBL | European Molecular Biology Laboratory |
| EMEM | Eagles Minimum Essential Medium |
| EPO | Erythropoietin |
| ER | Endoplasmic Reticulum |
| ESTs | Expression Sequence Tags |
| EXP | Exponential |
| F | F-distribution = mean squares between effects / mean squares within effects |
| FBS | Foetal Bovine Serum |
| FITC | Fluorescein Isothiocyanate |
| G | Guanine |
| GA-1000 | Amphotericin-B |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GBM | Glioblastoma Multiforme |
| GENBANK | NIH genetic sequence database |
| Glu | Glutamic acid |
| GLUT-2 | Glucose transporter 2 |
| GOS-3 | Grade II/III oligodendroglioma cell line |
| Grp75 | Heat shock 70kDa protein 9 (mortalin) |
| H | Hours |

| | |
|------------------|-------------------------------------|
| H ₂ O | Water |
| HDJ1 | DnaJ (Hsp40) homolog |
| HepG2 | Hepatocellular carcinoma cell line |
| HIF-1 α | Hypoxia-inducible factor 1 alpha |
| HIF-2 α | Hypoxia-inducible factor 2 alpha |
| HIF-3 α | Hypoxia-inducible factor 3 alpha |
| HIF-1 β | Hypoxia-inducible factor 1 beta |
| HNF-3/fork | Hepatocyte nuclear factor 3/fork |
| HR | Heptad repeats |
| HR-A | Heptad repeats amino terminal |
| HR-B | Heptad repeats carboxy terminal |
| HR-C | Heptad repeats N terminus |
| HRE | Hypoxia responsive element |
| HSBP1 | Heat shock factor binding protein 1 |
| HSEs | Heat shock elements |
| HSF | Heat shock factor |
| HSF1 | Heat shock factor 1 |
| HSF2 | Heat shock factor 2 |
| HSF3 | Heat shock factor 3 |
| HSF4 | Heat shock factor 4 |
| HSP10 | Heat shock protein 10 |
| HSP22 | Heat shock protein 22 |
| HSP23 | Heat shock protein 23 |
| HSP26 | Heat shock protein 26 |
| HSP27 | Heat shock protein 27 |

| | |
|----------------|---|
| HSP40 | Heat shock protein 40 |
| HSP60 | Heat shock protein 60 |
| HSP68 | Heat shock protein 68 |
| HSP70 | Heat shock protein 70 |
| HSP70-1 | Heat shock protein 70-1 |
| HSP70-2 | Heat shock protein 70-2 |
| HSP70-hom | Heat shock protein 70-hom |
| HSP75 | Heat shock protein 75 |
| HSP90 | Heat shock protein 90 |
| HSP90 α | Heat shock protein 90 alpha |
| HSP90 β | Heat shock protein 90 beta |
| HSP90N | Heat shock protein 90kDa alpha (cytosolic), class A member 1 |
| HSPA | Heat shock protein 70 |
| HSPA1A | Heat shock 70kDa protein 1A |
| HSPA1B | Heat shock 70kDa protein 1B |
| HSPA1L | Heat shock 70kDa protein 1L |
| HSPA2 | Heat shock 70kDa protein 2 |
| HSPA3 | Heat shock 70kDa protein 3 |
| HSPA4 | Heat shock 70kDa protein 4 |
| HSPA4L | Heat shock 70kDa protein 4-like |
| HSPA5 | Heat shock 70kDa protein 5 |
| HSPA6 | Heat shock 70kDa protein 6 |
| HSPA7 | Heat shock 70kDa protein 7 |
| HSPA8 | Heat shock 70kDa protein 8 |

| | |
|---------|---|
| HSPA9 | Heat shock 70kDa protein 9 |
| HSPA9B | Heat shock 70kDa protein 9 (mortalin) |
| HSPA12A | Heat shock 70kDa protein 12A |
| HSPA12B | Heat shock 70kDa protein 12B |
| HSPA14 | Heat shock 70kDa protein 14 |
| HSPB | Family of small heat shock proteins |
| HSPB1 | Heat shock protein 27 |
| HSPC | Heat shock protein 90 |
| HSPC1A | Heat Shock Protein 90 alpha |
| HSPC2A | Heat Shock Protein 90 beta |
| HSPC3A | Heat shock protein 90kDa alpha (cytosolic), class A member 1 |
| HSPC5A | Heat shock protein 75, TNFR-associated protein 1 |
| HSPD | Heat shock protein 60 |
| HSPE | Heat shock protein 10 |
| HSPH1 | Heat shock 105kDa/110kDa protein 1 |
| HSR | Heat Shock Response |
| HYOU1 | Hypoxia up-regulated protein 1 |
| IDDM | Insulin Dependant Diabetes Mellitus |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| i-NOS | Inducible nitric oxide synthase |
| KCL | Potassium chloride |
| KDa | Kilo Dalton |

| | |
|-------------------|--|
| L-G | L-Glutamine |
| LiCl | Lithium Chloride |
| Lys | Lysine |
| M | Molar |
| MAP-KAPK-2 | Map kinase activated protein 2 |
| MAP-KAPK-3 | Map kinase activated protein 3 |
| MD | Mean Differences |
| Met | Methionine |
| mg | Milligrams |
| MgCl ₂ | Magnesium Chloride |
| MHC-III | Major Histocompatibility Complex Class 3 Region |
| min | minutes |
| ml | Milli-litres |
| mM | Micro molar |
| MRI | Magnetic Resonance Imaging |
| mRNA | Messenger RNA |
| mtDNA | Mitochondrial DNA |
| NaOH | Sodium hydroxide |
| NBD | N-Terminal Binding ATPase Domain |
| NCBI | National Centre for Biotechnology Information |
| NEAA | Non essential amino acids |
| NEF | Nucleotide Exchange Factor |
| ng | Nanograms |

| | |
|------------------|--|
| -NH ₂ | Amine group |
| NHA | Normal Human Astrocyte cell line |
| NIDDM | Non-Insulin Dependent Diabetes Mellitus |
| NTC | No Template Control |
| OCT | Optimal Cutting Temperature |
| OD | Optical density |
| P | Significant value between 0.0 and 1.0 |
| P7056k | P70 S6 kinase |
| PAS | Acronym for Per, ARNT and Sim |
| PBS | Phosphate Buffered Saline |
| PCK7 | YE1389 putative serine protease |
| PCR | Polymerase chain reaction |
| Per | Period homolog |
| pg | Picograms |
| Phe | Phenylalanine |
| PI | Propidium Iodide |
| PKD1 | Polycystin-1 |
| PMI | Post Mortem Interval |
| qRT-PCR | Quantitative RT-PCR |
| rhEGF | Recombinant Human Epidermal Growth Factor |
| RNA | Ribonucleic Acid |
| RPM | Revolutions per minute |
| rRNA | Ribosomal Ribonucleic Acid |
| RT-PCR | Real Time Polymerase Chain Reaction |

| | |
|---------------|---|
| s | Seconds |
| SBD | Substrate Binding Domain |
| SD | Standard deviation |
| Sim | Single minded homolog |
| SMA560 | Murine anaplastic astrocytoma cell line |
| SMPs | Streptavidin Coated Magnetic Particles |
| SNPs | Single nucleotide Polymorphisms |
| snRNP | Small Nuclear Ribonucleoproteins |
| ssDNA | Single stranded DNA |
| ssRNA | Single Stranded RNA |
| STCH | Heat shock protein 70kDa family, member 13 |
| STZ | Streptozotocin |
| SwissProt | Part of the Swiss Institute of Bioinformatics (SIB) |
| T98G | Human glioblastoma cell line |
| t | $T \text{ value} = (\text{mean} - 1) / (\text{SD} / \text{square root of the number of samples})$ |
| T | Tyrosine |
| TAE | Tris-acetate-EDTA |
| <i>Taq</i> | <i>Thermus aquaticus</i> |
| TBE | Tris-borate-EDTA |
| Thr | Threonine |
| Tm | Melting temperature |
| TNF- α | Tumour Necrosis Factor Alpha |

| | |
|--------|------------------------------------|
| Trap1 | TNFR-associated protein 1 |
| tRNA | Transfer Ribonucleic Acid |
| U87-MG | Grade IV glioblastoma cell line |
| UTR | Untranslated Region |
| UV | Ultra Violet |
| v/v | Volume/volume |
| w/v | Weight/volume |
| VEGF | Vascular endothelial growth factor |
| VHL | Von hippel-lindau |
| WHO | World Health Organisation |
| WWW | World Wide Web |

Chapter 1

Introduction

1.1 Molecular Chaperones and Heat Shock Proteins

Protein synthesis is a complex molecular process which has been reviewed recently by Babitzke *et al*, (2009); Braakman and Bulleid, (2011) and Zamecnik, (2005). Translation involves interactions between three major groups of RNA molecules; ribosomal ribonucleic acid (rRNA), transfer ribonucleic acid (tRNA) and messenger ribonucleic acid (mRNA) templates, and a large number of accessory protein factors. Eukaryotic protein synthesis occurs in the cytoplasm at ribosomes, where the genetic information encoded by mRNA molecules is translated, according to the genetic code, into a corresponding sequence of amino acids to form a polypeptide.

Amino acids are brought to the peptide site of a ribosome by charged tRNA molecules, and the complementary anticodon in the tRNA specifically binds to the codon in the mRNA. Protein factors or initiation factors are required during the initiation phase of protein synthesis, interacting with the ribosome-mRNA complex, and dissociate when polypeptide chain synthesis is initiated. After the initiation complex has formed, elongation of the polypeptide chain proceeds. Elongation begins when the carboxyl, (–COOH) group of an amino acid carried by a charged aminoacyl-tRNA (aa-tRNA), binds to the peptidyl site of the ribosome with the amino, (NH₂) group of the amino acid of another aa-tRNA in the aminoacyl site. The peptidyl tRNA translocates from the aminoacyl site to the peptide site where the tRNA disassociates from the peptide to the exit site. This process repeats until a nonsense codon is reached. Termination of this process requires specific protein release factors which recognise nonsense codons, causing the nascent polypeptide chain to release from the peptidyl tRNA and the ribosome.

The polypeptide that emerges from the ribosome is inactive and must undergo protein folding, one of four post translation processes, before taking on its functional role in the cell.

Proteins are generally considered to have four distinct levels of structural organization.

The primary structure of a protein is formed by the linkage of amino acids via covalent peptide bonds to form a linear polypeptide chain. The two ends of the polypeptide chain are chemically distinct; the start of the polypeptide chain has a free amino group which is referred to as the N-terminus, and the other end of the polypeptide chain has a free carboxyl group, termed the C-terminus (Braakman and Bulleid, 2011).

The secondary structure of proteins describes the folding and twisting of the linear polypeptide chain into specific conformations, such as α -helix and β -pleated sheet structures. The α -helix structure is formed by intramolecular hydrogen bonding between the -NH groups of one amino acid and the -COOH groups of an amino acid that is four amino acids away within the chain. Repeated formation of this bonding results in the helical coiling of the polypeptide chain. The β -pleated sheet structure is formed by intermolecular hydrogen bonding between the amide hydrogen of one chain to the amide oxygen of a neighbouring chain resulting in a zigzag or pleated formation (Braakman and Bulleid, 2011).

Tertiary structures are formed through the folding of secondary structure into three-dimensional structures determined by a variety of bonding interactions, including hydrogen bonding (Arunan *et al*, 2011), salt bridges (Kumar and Kussinov, 2002), disulphide bonds (Sevier and Kaiser, 2002) and non-polar hydrophobic interactions (Rose *et al*, 2006) between the -side chains on the amino acids (Braakman and Bulleid, 2011).

The quaternary structure is formed by the clustering of two or more proteins linking together to form a multi-subunit protein, but it is worth noting that not all proteins exhibit quaternary structures. Again, a variety of bonding interactions, including hydrogen bonding, salt bridges, and disulphide bonds, hold the protein in its final structural shape, but also allow the proteins to revert back to their component polypeptide or change their subunit composition, depending on their functional requirement (Braakman and Bulleid, 2011).

Folding of newly synthesized proteins requires the interaction of numerous protein cofactors, referred to as molecular chaperones. Molecular chaperones ensure that polypeptide chains are not damaged during the transport and assembly stages of protein production. These proteins are not part of the final proteins with which they are associated. They are a diverse family of largely unrelated proteins (Ellis and Hemmingsen, 1989; Ellis and van der Vies, 1991; Feder, 1999; Hartl *et al*, 2011). Molecular chaperones maintain newly synthesized polypeptides in an unfolded state for translocation across intracellular membranes and hence recognise and selectively bind to new polypeptide chains and to partially folded intermediates of proteins (Atalay *et al*, 2009; ; Jolly and Morimoto 2000; Welch *et al*, 1993). They also prevent the aggregation and misfolding of newly synthesized proteins, prevent non-productive interactions with other cell components, direct the assembly of larger proteins and multiprotein complexes, and cause previously folded proteins to unfold during exposure to stressful conditions (Atalay *et al*, 2009; Ellis, 1993; Jolly and Morimoto, 2000; Welch *et al*, 1993).

Molecular chaperones play a crucial protective role under conditions of cellular stress and damage, preventing the appearance of folding intermediates that lead to either

misfolded or damaged protein molecules. When their production or activity is induced in response to either acute or chronic stress, molecular chaperones capture folding intermediates and prevent their misfolding or premature aggregation. They are also implicated in the control of cellular apoptosis (Ellis, 1993; Jolly and Morimoto, 2000; Welch *et al*, 1993).

Molecular chaperones are highly conserved groups of proteins, which comprise a number of unrelated families, and most are classified as heat shock (stress) proteins. They are highly ubiquitous and are found in both prokaryotic and eukaryotic cells, where they can be found localized in the cytoplasm, nucleus, endoplasmic reticulum or the mitochondria. The existence of these proteins, and their associated genes, was discovered from studies of the response to cells to heat shock, first reported by Ferruccio Ritossa in 1962. This was first observed in the salivary gland cells of the fruit fly *Drosophila busckii*, which were exposed to an elevated temperature of 37°C for 30 min, followed by recovery at their normal temperature of 25°C. Ritossa described a unique set of chromosomal ‘puffs’ (Figure 1.1) observed after exposure of these cells to heat, together with the increased production of then unknown proteins with molecular masses of 70 and 26 KDa (Ritossa, 1962; Ritossa, 1963; Trivedi *et al*, 2010). The polytene chromosomes present in *Drosophila* salivary gland cells allowed the observation of ‘puffing’ of specific regions of the chromosomes in response to heat shock, which resulted from high levels of transcription of heat shock genes.

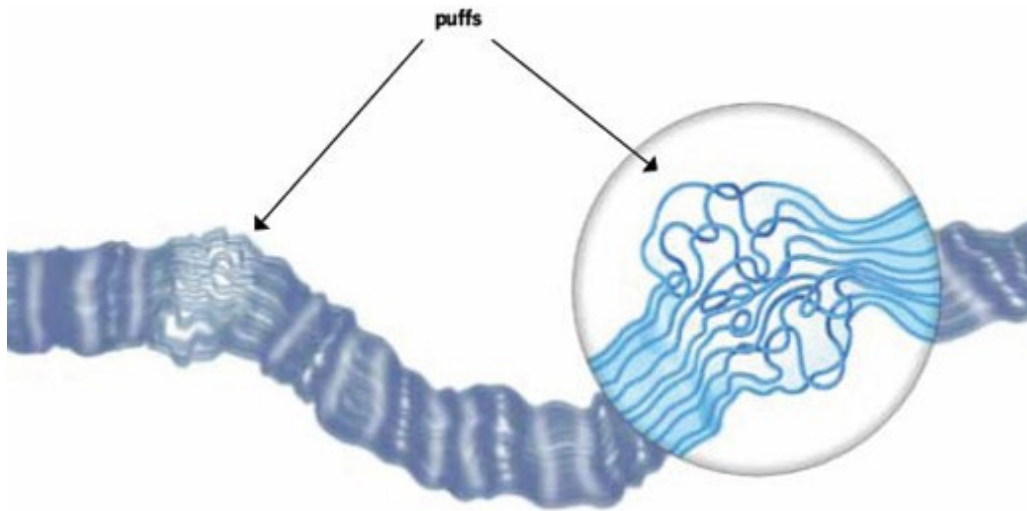


Figure 1.1. Representation of a light microscope image of Ritossa's chromosomal puffs, which are characterised by localized swellings of specific regions of a polytene chromosome. These 'puffs' result from localised synthesis of RNA, resulting from high levels of transcription of heat shock genes at each chromosomal location (Morimoto, 2010).

Over the next decade, further studies showed that these chromosomal puffs were also induced by a variety of other cellular treatments, such as exposure to dinitrophenol, sodium salicylate (Ritossa, 1962), actinomycin D (Berendes, 1968), or anoxia (Ashburner, 1970); these treatments all resulted in cellular stress. The production of puffing was shown to be associated with newly synthesized RNA, indicating that these resulted from high levels of gene expression (Leenders and Berendes, 1972; Ritossa, 1962) and that the response was rapidly induced, as puffs were produced within a few minutes of treatment (Ashburner, 1970; Berendes, 1968). The phenomenon was also found in other *Drosophila* species such as *Drosophila melanogaster*, *Drosophila hydei* and *Drosophila similans* (Berendes, 1965; Ritossa, 1964). Later studies by Guttman and Gorovsky, (1979); Kelly and Schlesinger (1982); McAlister *et al*, (1979); Miller *et al*, (1979) and Neidhardt *et al*, (1984) showed that this was a wide-spread phenomenon in most other organisms, indicating that heat shock response was a fundamental cellular function. It was discovered that heat and various other types of stress could induce the synthesis of similar proteins in yeast (McAlister *et al*, 1979; Miller *et al*, 1979); cultured avian cells (Kelly and Schlesinger, 1982); tetrahymena (Guttman and Gorovsky, 1979) and *Escherichia coli* (Neidhardt *et al*, 1984). Studies undertaken by Ashburner and Berendes (1978); Choa and Guild, (1986) and Huet *et al*, (1993) have shown sequential activation of early and late puffs in the polytene chromosomes in multiple gene sets during heat shock. Early puffs are active for approximately 4 hours and then regress, during which time late puff sets become active (Ashburner, 1970; Ashburner and Berendes, 1978; Berendes, 1968; Leenders and Berendes, 1972). In *Drosophila melanogaster*, nine puffs induced by heat shock in polytene nuclei were identified on different chromosomes; the left arm of chromosome 2 loci 33B, left arm of chromosome 3 loci 63BC, 64EF, 67B, right arm of chromosome 3 loci 70A, 87A, 87C, 93D and 95D (Ashburner, 1970; Mukherjee and Lakhotia,

1979). The first gene products resulting from these chromosomal puffs were identified some 12 years later and subsequently termed –heat shock proteins| (Neuer *et al*, 2000; Tissiere *et al*, 1974). In both eukaryotic and prokaryotic organisms, heat shock protein genes are scattered at various chromosomal locations, with some related genes clustered or, if unrelated, interspersed with independently regulated genes. On the right arm of chromosome three, five copies per haploid genome for the gene HSP70 are present, two at locus site 87A and three at locus site 87C, also the gene for HSP68 is present at locus site 95D (Holmgren *et al*, 1979). On the left arm of chromosome three, four of the small heat shock proteins are encoded at locus site 67B, in the order HSP27, HSP23, HSP26 and HSP22 (Corces *et al*, 1980; Craig and McCarthy, 1980; Voellmy *et al*, 1981) and also HSP83 coding sequences are located at locus 63BC.

The normal growth temperature for *Drosophila* cells is 25°C. When the cells are subjected to heat shock with temperatures between 29 – 38°C, heat shock proteins are induced with a maximum response by temperatures between 36 – 37°C (Lindquist, 1980). Within minutes of temperature elevation, heat shock mRNAs appear in the cytoplasm and are translated immediately with high efficiency (Lindquist, 1980). Within an hour of heat shock, several thousand heat shock transcripts are found in each cell (Lindquist, 1980). During this time, translation of pre-existing messages (Kelly and Schlesinger, 1982; Lindquist, 1981) and transcription of previously active genes are repressed (Berendes, 1968; Findly and Pederson, 1981; Lindquist, 1986). If the cells are maintained at elevated temperatures, heat shock proteins will continue to be the primary products of protein synthesis. If the cells are returned to their normal growth temperature, normal protein synthesis will be gradually resumed, the timing of which is dependent on the severity of the heat shock induced (DiDomenico *et al*, 1982).

In other organisms, induction of heat shock protein genes is equally rapid. However, the maximum induction temperature varies depending on the physiological growth temperature. For example, in humans, with a normal physiological temperature of 37°C, the maximum response temperature is 42°C; in yeast, with a normal physiological temperature of 30 – 37°C, the maximum response temperature is 39 - 40°C (Lindquist *et al*, 1982), in salmon/trout, with a normal physiological temperature of 5 – 15°C (Kothary and Candido, 1982), the maximum response temperature is 28°C and in *E.coli*, with an optimal growth temperature of 37°C, the maximum response temperature is 45 – 50°C (Neidhardt *et al*, 1984; Yamamori *et al*, 1978). In organisms that grow over a broad range of temperatures, the maximum response temperature is generally 10 – 15°C above the optimum physiological temperature, whereas for organisms/species that grow over a more restricted temperature range, the maximum response temperature is generally 5°C above the optimum physiological temperature (Lindquist, 1986).

In some organisms, heat shock response appears to be either transient or sustained. For example, in *E.coli* heat shock response is transient when the normal growth temperature is raised to 42°C but is sustained when the temperature is raised to 45 - 50°C (Neidhardt *et al*, 1984; Yamamori *et al*, 1978). In most organisms heat shock will be transient at moderate temperatures where normal growth will be resumed, and sustained at higher temperatures until the cells slowly begin to die (Lindquist, 1986).

The majority of cells in multicellular organisms respond to heat shock. For example, cells respond identically in different tissues of *Drosophila* such as the malpighian tubules, imaginal wing discs, brain, salivary glands, and tissue cultured cells (Tissiere *et al*, 1974), and in *Rattus norvegicus* heart, brain, liver, lung, kidney, thymus and adrenal gland tissues, the same phenomenon is observed (White and Currie, 1982). However,

there are some exceptions. For example, in *Rattus norvegicus*, *Hsp70* cannot be induced in the brain until three weeks postpartum (Tissiere *et al*, 1974).

It is worth noting that, due to the inconsistency in nomenclature that has been used for heat shock genes and their products in published literature, regarding abbreviations, gene and protein symbols in different species, there was a need for standardization. The use of different names for these genes and proteins make comparison of different studies very difficult. For the purposes of this thesis, nomenclature for human and non-human primates has been based on the Guidelines for Human Nomenclature (Show *et al*, 1987). Other species-based nomenclature, such as mouse, rat and chicken species, has been based on the Rules for Nomenclature of Genes (<http://www.informatics.jax.org/>).

For human / non-human primates and other species, nomenclature for proteins will be non-italicized and in lower case, and will be italicized if referring to genes. Gene symbols for humans and non-human primates are designated by a combination of italicized upper case letters and Arabic letters, e.g. *HSPA*. Protein designations are the same as for the gene symbol but not italicized and all in upper case e.g. HSPA.

For mouse, rat and chicken, nomenclature will be non-italicized and in lower case, and will be italicized if referring to genes. Gene symbols are designated by a combination of italicized letters and Arabic letters, the first letter in upper case and the rest lower case: e.g. *HSPA*. Protein designations are the same as for the gene symbol, but not italicized and all in upper case: e.g. HSPA.

In mammalian cells, heat shock proteins function as either molecular chaperones or proteases (Jolly and Morimoto, 2000) and are classified into five major families, according to their molecular size or function. These are HSP90, HSP70, HSP60, HSP40 and HSP27 (Table 1.1 Craig *et al*, 1994; Jolly and Morimoto, 2000; Kampinga *et al*, 2009; Lindquist and Craig, 1988; Morimoto *et al*, 1994; Powers *et al*, 2007; Sreedhar *et al*, 2004). These families are described in more detail later in this chapter in section 1.3.

Table 1.1 Major Heat Shock Proteins (Taken from Craig *et al*, 1994; Jolly and Morimoto, 2000; Kampinga *et al*, 2009; Lindquist and Craig, 1988; Morimoto *et al*, 1994; Powers *et al*, 2007; Sreedhar *et al*, 2004)

| Heat shock protein Families | Co-chaperones or Isoforms | Expression | Localization | Activity / Function |
|-----------------------------|---------------------------|-------------------------|--------------------|---|
| HSP27 | Various | Constitutive /Inducible | Cytoplasm/ Nucleus | Prevent heat denaturation protein aggregation via ATP-independent formation of high molecular weight oligomers; phosphorylation of HSP27 monomers/dimers regulate microfilament polymerization. |
| HSP40 | Hdj1 and Hdj2 | Constitutive /Inducible | Cytoplasm/ Nucleus | Cochaperone activity with HSP70 proteins. Regulates adenosine triphosphate (ATP) activity and substrate release. |
| HSP60 | mtHsp60/HSP10-mito | Constitutive /Inducible | Mitochondria | Folds newly imported mitochondrial proteins. |
| | TRIC/CCT | Constitutive /Inducible | Cytoplasm | Folds approx. 10% of cytosolic polypeptide chains downstream of the Hsp70 machinery. |
| HSP70 | HSC70 | Constitutive /Inducible | Cytoplasm /Nucleus | Cognate form assists constitutive folding and transport of proteins to organelles. |
| | HSP70.1 | Constitutive /Inducible | Cytoplasm | Induced upon heat shock and mediate similar functions in response to stress-induced increase in protein misfolding and aggregation |
| | HSP70.2 | Constitutive | Cytoplasm | |
| | HSP70.3 | Constitutive | Cytoplasm | |
| | mtHsp70/Grp75 | Constitutive | ER/Cytoplasm | Protein folding and translocation in mitochondria |
| | Bip/Grp78 | Constitutive /Inducible | ER/Cytoplasm | Binds folding and translocation intermediates to prevent aggregation |
| HSP90 | HSP90- α | Inducible | Cytoplasm /Nucleus | Growth promotion, cell cycle regulation, stress induced cytoprotection, signal transduction |
| | HSP90- β | Constitutive | Cytoplasm /Nucleus | Cellular transformation, signal transduction, cytoskeletal stabilization, long term cell adaption |
| | HSP-N | Constitutive /Inducible | Cytoplasm /Nucleus | Cellular transformation |
| | HSP75 1TRAP-1 | Constitutive /Inducible | Mitochondria | Cell cycle regulation |

Due to the expanding number of members of the human heat shock protein families and the inconsistency in their nomenclature, it was suggested that a more consistent and standardized nomenclature should be assigned. In recently reviewed literature names used for the human heat shock protein family members have been inconsistent with a number of different names and symbols being used for the same gene product.

In 2009, the nomenclature for human heat shock protein families and the human chaperonin families were revised from previous designations (Kampinga *et al*, 2009). The new guidelines for the nomenclature propose HSPC (HSP90), HSPA (HSP70), DNAJ (HSP40), HSPB (small HSP) and HSPD/E (HSP60/HSP10) respectively. The new nomenclature is mainly based on the systematic gene symbols that have been assigned by the HUGO Gene Nomenclature Committee that are used as primary identifiers in gene databases such as the National Centre of Biotechnology Information (NCBI), Entrez Gene and Ensembl.

Throughout this thesis, the new nomenclature described above for (example HSPA/HSP70) has been used for all the heat shock protein families reported (Table 1.2).

Table 1.2. Revised nomenclature for the heat shock protein families used in this study (taken from Kampinga *et al*, 2009)

| Family | Gene | Protein Name | Previous Nomenclature |
|--------|---------------|--------------|----------------------------|
| HSPB | | | Small heat shock proteins |
| | <i>HSPB1</i> | HSPB1 | HSP27 |
| DNAJ | | | HSP40 |
| | <i>DNAJA1</i> | DNAJA1 | Hdj2, HSP40 |
| | <i>DNAJB1</i> | DNAJB1 | Hdj1, HSP40 |
| HSPD | | | HSP60 |
| | <i>HSPD1</i> | HSPD1 | HSP60 |
| HSPA | | | HSP70 |
| | <i>HSPA1A</i> | HSPA1A | HSP70-1, HSP72, HSP70.3 |
| | <i>HSPA1B</i> | HSPA1B | HSP70-2 |
| | <i>HSPA5</i> | HSPA5 | BIP, GRP78 |
| | <i>HSPA8</i> | HSPA8 | HSC70, HSP71, HSP71, HSP73 |
| | <i>HSPA9</i> | HSPA9 | GRP75, mtHSP70 |
| HSPC | | | HSP90 |
| | <i>HSPC1a</i> | HSPC1 | HSP90- α |
| | <i>HSPC2a</i> | HSPC2 | Hsp90- β |
| | <i>HSPC3a</i> | HSPC3 | HSP-N |
| | <i>HSPC5a</i> | HSPC5 | HSP75, TRAP-1 |

Each family of heat shock proteins contains members which are expressed either constitutively or which are induced in response to stimuli and are targeted to different sub-cellular compartments (Garrido *et al*, 2006; Powers *et al*, 2009). Both constitutive and inducible heat shock proteins carry out numerous functions including: nascent protein folding; prevention of the formation of protein aggregates; assisting in the re-folding of denatured proteins; facilitation of degradation of irreparable proteins; modulation of the assembly or disassembly of protein complexes and assisting in the translocation of proteins across cellular membranes (Calderwood *et al*, 2006; Mosser and Morimoto, 2004; Powers *et al*, 2009). In addition to the above roles, heat shock proteins also play a strong part in cytoprotection and allow cells to adapt to gradual changes in their environment and to survive under hostile or otherwise lethal conditions (Calderwood *et al*, 2006; Mosser and Morimoto, 2004; Parcellier *et al*, 2003; Powers *et al*, 2009). The expression and functions of heat shock protein genes have been extensively studied in a wide variety of tissues and cell types that have been exposed to a range of stress conditions. Although the production of heat shock proteins results in the protection of cells from the effects of further stresses, they are deemed toxic if present in the cells for any prolonged period (Theodorakis *et al*, 1999).

Heat shock proteins are thus a family of highly conserved ubiquitous proteins encoded by genes which are activated, not only in response to various physiological and environmental stress conditions (Figure 1.2.), but also in cells affected by various diseases, such as cancer, Alzheimer's, Parkinson's and Huntington's diseases and diabetes, and by fever or inflammation (Jolly and Morimoto, 2000; Lindquist, 1986; Morimoto, 1993).

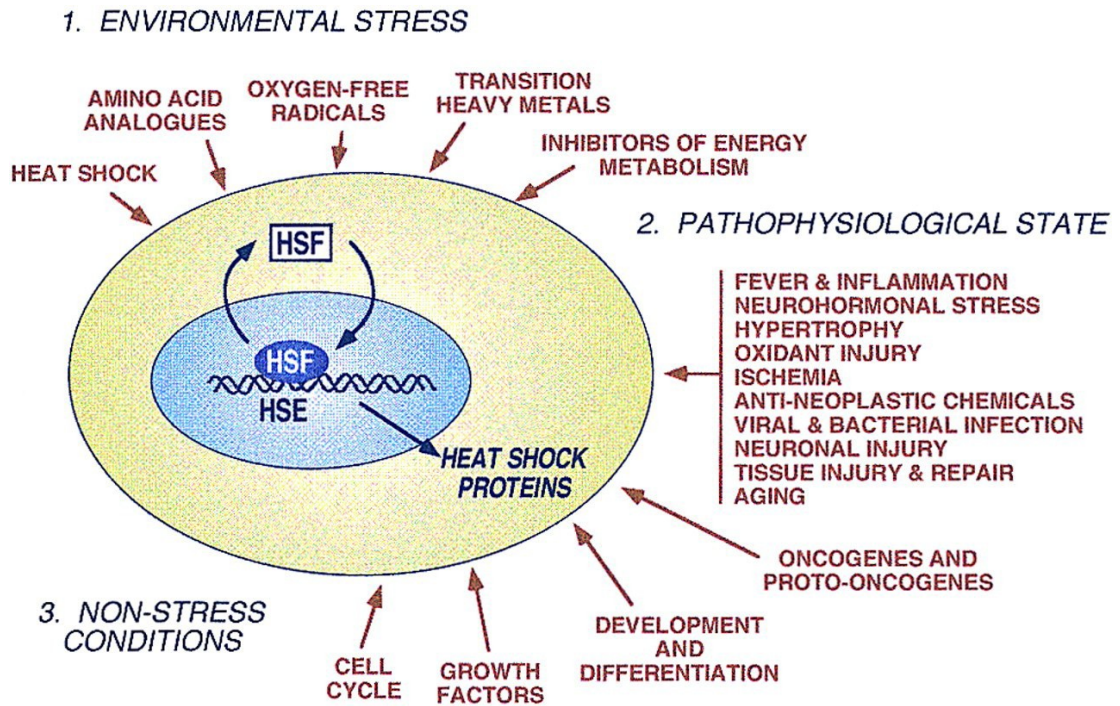


Figure. 1.2. Diagram showing the conditions that induce heat shock in response to environmental, physiological and non-stressful conditions (Taken from Morimoto, 1998).

Heat shock proteins achieve this by recognizing nascent polypeptides, partially and misfolded regions of proteins and exposed hydrophobic regions of amino acids (Figure 1.3) (Bakau and Horwich, 1998; Jolly and Morimoto, 2000; Morimoto, 1998; Nollen and Morimoto, 2002; Parsell and Lindquist, 1993). Under normal growth conditions nearly all heat shock proteins are constitutively expressed at relatively low but constant levels, as their normal cellular function is to maintain protein homeostasis by regulating correct protein folding (Hartl and Hayer-Hartl, 2002; Nollen and Morimoto, 2002). However, under conditions of stress, induction of heat shock gene expression results in heat shock proteins being produced at much higher levels to prevent incorrect polypeptide aggregation and protein denaturation during physiochemical insults to enhance cell survival. Constitutive heat shock proteins perform housekeeping functions by acting as molecular chaperones. They assist polypeptides to achieve their proper conformation by binding to nascent proteins via their C-terminal domain (Mosser and Morimoto, 2004; Parsell and Lindquist, 1994).

The response of cells to such stress conditions is dependent on the concentration of heat shock proteins in the cell prior to stress, and on the severity of the stress, based upon its intensity and duration. The outcome of such stress events is either cell survival or apoptotic or necrotic cell death (Mosser and Morimoto, 2004). Thus, heat shock proteins play a role in the cell cycle. Elevated levels of heat shock proteins are seen in cells recovering from induced stress, and these cells are in a cytoprotected state, protecting them from further exposures to stress conditions (Nollen and Morimoto, 2002). Cells initiating apoptosis after induced stress also elicit the production of heat shock proteins. This continues until such time as heat shock protein levels in the cell have returned to normal (Mosser and Morimoto, 2004; Parsell and Lindquist, 1993).

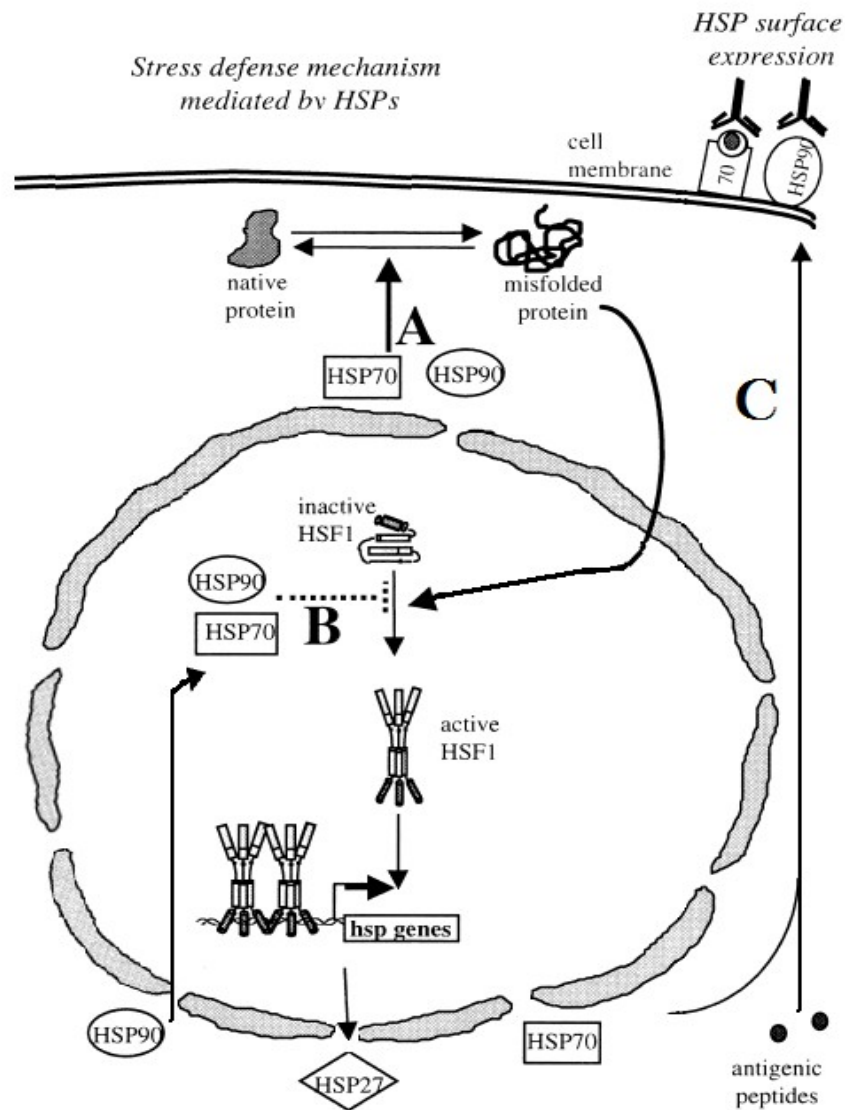


Figure 1.3 Schematic diagram showing the stress defence mechanism mediated by heat shock proteins. Stress such as heat shock, hypoxia, post-mortem and cancer are associated with the emergence of misfolded proteins. Initially heat shock proteins will assist misfolded proteins and folded intermediates to obtain a native state (pathway A), resulting in the activation of the heat shock transcription factor in the nucleus, which activates the transcription of heat shock protein genes (pathway B). Heat shock proteins are also involved in the negative regulation of their own synthesis by autoregulation (pathway B). In some tumour cells heat shock proteins have been shown to be expressed at the cell surface (pathway C) (Taken from Jolly and Morimoto, 2000).

The induction of heat shock gene expression in response to stress conditions is dependent on a specific DNA sequence, termed the heat-shock element (HSE). The HSE is a stress responsive operator element located in the promoter regions of heat shock genes (Fernandes *et al*, 1994; Morimoto *et al*, 1994; Wu *et al*, 1994). It consists of a series of inverted pentameric units with the nucleotide sequence 5' nGAAn 3' (Santoro, 2000). Inducible transcription of heat shock protein genes require the *de novo* binding of the HSE by a heat shock transcription factor (HSF), which is constitutively produced.

In mammalian cells, the HSF gene family consists of the four members *HSF1*, *HSF2*, *Hsf3* and *HSF4* (Table 1.3), of which three (*HSF1*, *HSF2* and *HSF4*) have been characterized in human cells. The HSF1 protein is ubiquitously synthesised and plays a major role in the stress induced expression of heat shock protein genes (de Thonel *et al*, 2011). The HSF2 protein is activated during specific stages of cell development and is associated with development of the brain and reproductive organs (de Thonel *et al*, 2011). The HSF4 protein acts as an inhibitor of stress-induced gene expression (de Thonel *et al*, 2011; Jolly and Morimoto, 2000; Morimoto, 1993; Wu, 1995). The *Hsf3* gene has only been characterized in avian species and more recently identified in mouse as an orthologue of the chicken *Hsf3* gene (de Thonel *et al*, 2011; Nakai, 1999; Nakai and Morimoto, 1993; Tanabe *et al*, 1998). In mouse and avian species the HSF3 protein has been identified as a redundant heat shock responsive factor which is co-expressed with HSF1 suggesting that both HSF1 and HSF3 are involved in the activation of heat shock protein genes during heat shock (Tanabe *et al*, 1998).

Table 1.3. Characterization of heat shock factors across species (adapted from de Thonel *et al*, 2011; Morimoto, 1998).

| | HSF1 | | HSF2 | | HSF3 | | HSF4^(a) |
|---------------------------------|---|-----------------|---|-------------------------|------------------------------------|-----------------|--|
| Species | <i>Homo sapiens, Mus musculus, Rattus norvegicus, Gallus gallus</i> | | <i>Homo sapiens, Mus musculus, Rattus Norvegicus, Gallus gallus</i> | | <i>Mus musculus, Gallus gallus</i> | | <i>Homo sapiens Mus musculus, Rattus Norvegicus, Gallus gallus</i> |
| Homology between species | 92% | | 92% | | | | |
| Tissue Specific Location | Ubiquitous | | Ubiquitous | | Ubiquitous | | Tissue specific – heart, brain, pancreas, skeletal muscle |
| In vivo conditions | 37°C | 42°C heat shock | 37°C | 42°C heat shock | 37°C | 42°C heat shock | 37°C |
| Protein size | | | | | | | |
| Native (KDa) | 70 | 178 | 127 | 127 | | | |
| Denatured (KDa) | 70 | 85 | 72 | 72 | 69 | 69 | 55 |
| Localization | Cytoplasmic / Nuclear | Nuclear | Cytoplasmic and Nuclear | Cytoplasmic and Nuclear | Cytoplasmic | Nuclear | Constitutively Nuclear |
| Oligomeric State | Monomer | Trimer | Dimer | Dimer | Dimer | Trimer | Trimer |
| DNA-binding | | + | - | - | - | + | Constitutive DNA binds but lacks transcriptional activity |

(a) DNA – binding activity is lost *in vitro* upon heat shock

Heat shock protein synthesis is regulated at the transcriptional level by heat shock factors (HSF) which assist, not only in the long term induction of heat shock protein genes, but also in the regulation of gene expression and developmental processes (Morimoto, 1998; Sreedhar *et al*, 2004). All members of the heat shock factor protein family share two evolutionary conserved structural functional domains: a conserved DNA-binding domain (DBD) at the amino terminus and an oligomerization domain (Figure 1.4). The heat shock factor DBD is a member of the winged helix-turn-helix hepatocyte nuclear factor 3/fork (HNF-3/fork) transcription factors (de Thonel *et al*, 2011; Harrison *et al*, 1994, Vuister *et al*, 1994). The oligomerization domain comprises arrays of hydrophobic heptad repeats (HRs) which are divided into two subdomains: the amino terminal HR-A and the carboxy-terminal HR-B, both of which are essential for trimer formation (de Thonel *et al*, 2011; Peteranderl and Nelson, 1992; Shamovsky and Nudler, 2008; Soger and Nelson, 1989). A third HR domain (HR-C) is also located in *Drosophila* HSF and mammalian HSF1 which maintains heat shock factors in a monomeric state by suppressing trimer formation through interactions with HR-A/B. (de Thonel *et al*, 2011; Green *et al*, 1995; Shamovsky and Nudler, 2008; Shi *et al*, 1995; Wisniewski *et al*, 1996; Zuo *et al*, 1995).

Heat shock response involves the functional activation of heat shock factor 1 (HSF1), in response to damage to other cellular proteins which requires the action of molecular chaperones. HSF1 is present in both stressed and unstressed cells. Under normal conditions, HSF1 exists in the cytoplasm as an inert monomer, which is unable to bind to DNA and does not show any transcriptional activity (Pockley, 2003; Santoro 2000). HSF1 is constitutively phosphorylated and lacks the ability to bind to the cis-acting heat shock elements within the promoter regions of the heat shock protein genes (Wang *et al*, 2003, Wu, 1995). In a stressed state, when non-native or damaged proteins are

detected, induction of transcriptional activity by heat shock factor 1 requires that the inactive HSF1 monomer is converted to phosphorylated trimers which have the capacity to bind to DNA, and which also translocate from the cytoplasm to the nucleus (Baler *et al*, 1993; Pockley, 2003; Santoro 2000; Sarge *et al*, 1993; Westwood *et al*, 1993).

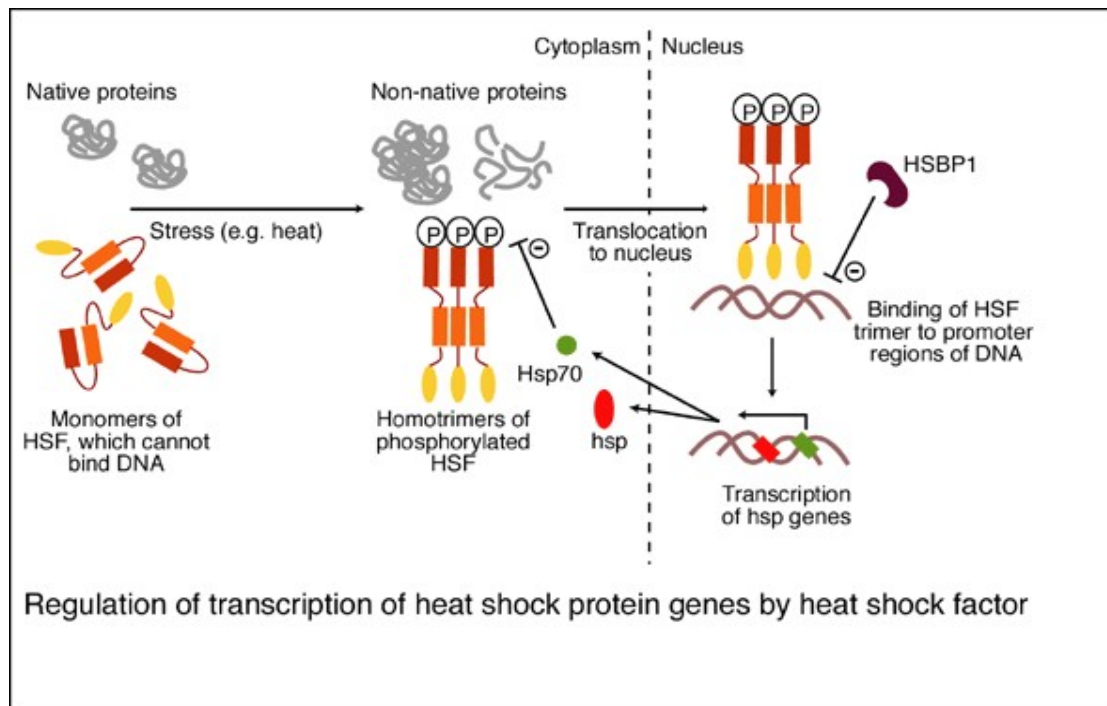


Figure 1.4. Regulation of transcription of heat shock protein genes by heat shock factor. Heat shock factor (HSF) is present in the cytoplasm in a monomeric state that is unable to bind to DNA. Under conditions of stress such as heat shock, the flux of non-native proteins leads to phosphorylation and trimerisation of HSFs. The trimers translocate to the nucleus, bind the promoter regions of heat shock protein genes and mediate heat shock protein gene transcription (Adapted from Pockley, 2003).

Activation induced trimerization of HSF1 occurs by the intermolecular coiled-coil interaction of the HR-A/B and HR-C domains (Figure 1.5) (Rabindran *et al*, 1993), which is mediated by leucine zipper domains (three hydrophobic heptad repeats of HR-A/B) in the N-terminus, which is in turn subject to intramolecular negative regulation by a fourth leucine zipper (hydrophobic heptad repeat of HR-C) in the C-terminus. The phosphorylated HSF1 binds to the heat shock element, thereby allowing transcription of the target genes (Santoro, 2000).

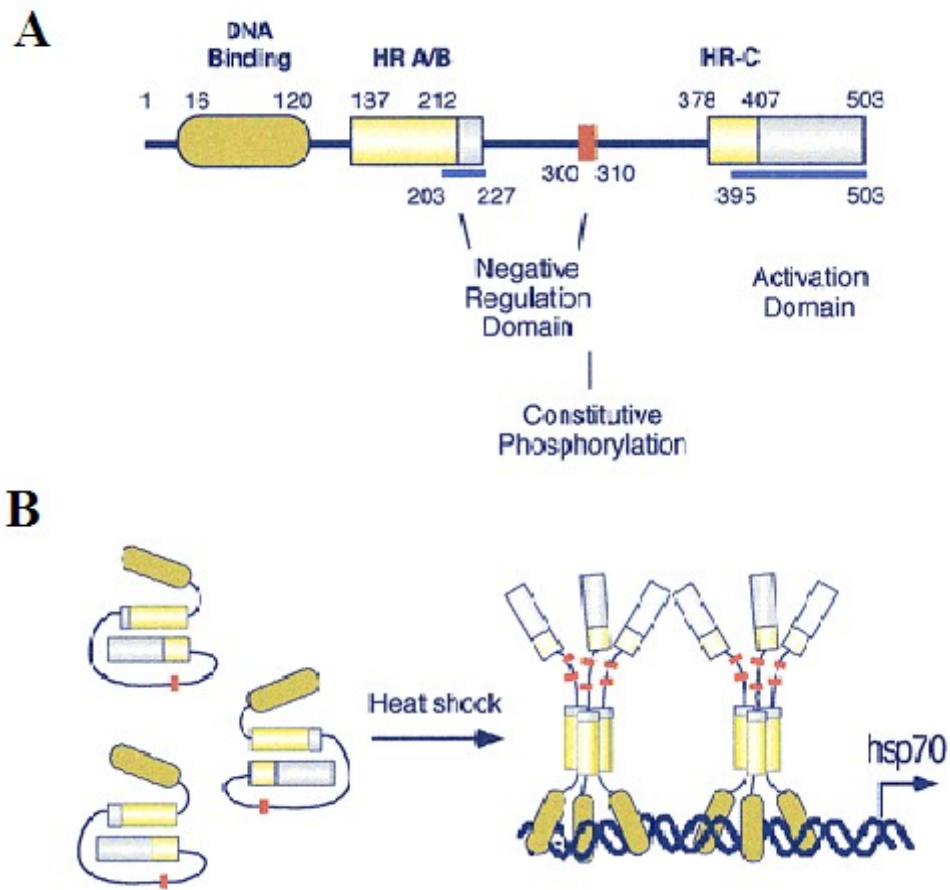


Figure 1.5. The general structural and regulatory features of HSFs. (A) A schematic representation of HSF1 structural motifs corresponding to the DNA-binding domain (DBD), hydrophobic heptad repeats (HR-A, HR-B and HR-C), the carboxyl-terminal transcriptional activation domain and the negative regulatory domains all of which influence HSF1 activity. (B) A schematic representation of the intramolecular negatively regulated monomer which, upon exposure to stress, becomes activated to form homotrimers with DNA-binding activity (adapted from de Thonel *et al*, 2011 and Santoro, 2000).

Various mechanisms for HSF binding and regulation of heat shock protein gene transcription have been proposed, and these are described here with respect to heat shock protein 70 gene (*HSPA/HSP70*) (Abravaya *et al*, 1992; Morimoto, 1993). In the absence of stress in cells, HSF is present in both the cytoplasm and the nucleus and is maintained in a non-DNA binding monomeric state through transient interactions with *HSPA/HSP70*, which prevents the activation of HSF (Abravaya *et al*, 1992; Morimoto, 1993). During heat shock, denatured and misfolded proteins accumulate and prevent new substrates for *HSPA/HSP70* which compete for *HSPA/HSP70* binding with HSF (Abravaya *et al*, 1992). This results in the release of HSF from *HSPA/HSP70*. The released HSF proteins oligomerises into a trimeric state which binds to the HSE sequence within the heat shock gene promoter region and becomes inducibly phosphorylated by active protein kinases, such as protein kinase A (PKAca) , at serine residues in the carboxyl-terminal domain, thus activating stress induced transcription (Abravaya *et al*, 1992; Morimoto, 1993; Zhang *et al*, 2011). Activated HSF induces transcription of the heat shock protein genes resulting in increased levels of synthesis of heat shock proteins within the cell. This eventually results in the formation of a new HSF-*HSPA/HSP70* complex (Abravaya *et al*, 1992), which the HSF-*HSPA/HSP70* complex dissociates from the HSE resulting in the cessation of transcription (Figure 1.6) (Abravaya *et al*, 1992; Morimoto. 1993).

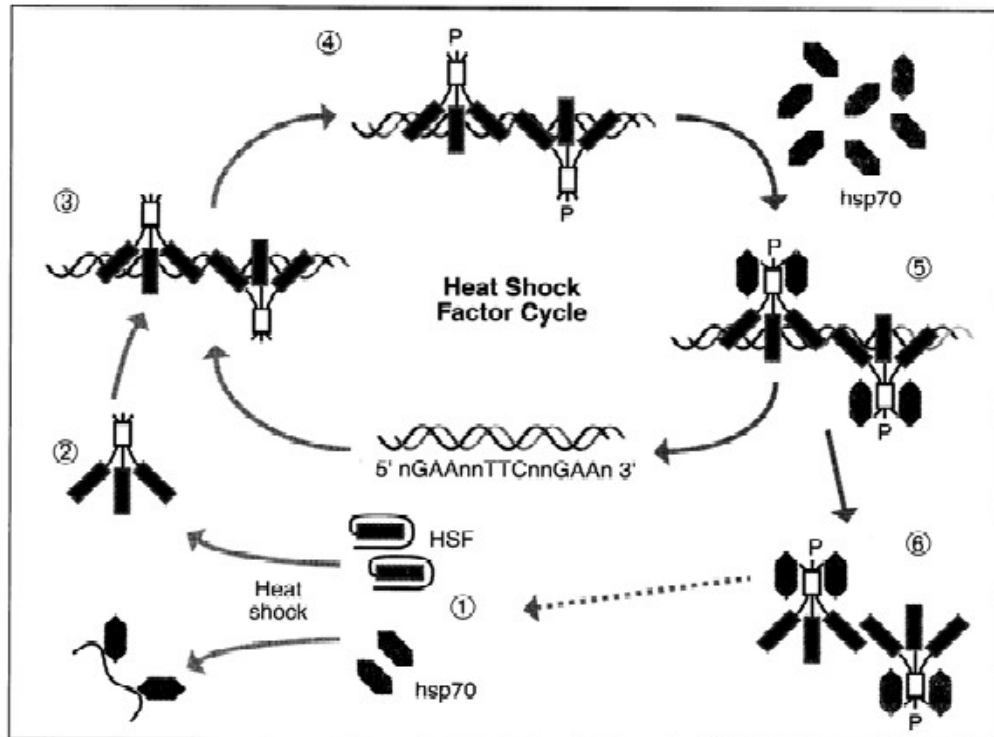


Figure 1.6. Heat shock factor regulation (adapted from Morimoto. 1993). In the absence of stress in cells, HSF is maintained in a non-DNA binding monomeric state (1). Following stress, HSF oligomerises into a trimeric state (2). HSF binds to specific sequences, HSE, in heat shock gene promoters (3), and becomes phosphorylated (4). Transcriptional activation of heat shock genes increases the levels of HSPA/HSP70 which forms a HSF-HSPA/HSP70 complex (5). HSF dissociates from the DNA and reverts back to a non-DNA binding monomer (Morimoto. 1993).

Complexes of HSPA/HSP70 and HSF trimers have been detected in cells and are consistent with the role of chaperones during attenuation of the heat shock transcriptional response (Abravaya *et al*, 1992; Baler *et al*, 1992; Morimoto, 1998). In the absence of stress, overexpression of *HSPA/HSP70* or *DNAJ/HSP40* prevents the inducible transcription of heat shock genes (Morimoto, 1998; Mosser *et al*, 1993; Shi *et al*, 1998). These molecular chaperones bind directly to the HSF1 transactivation domain. However, HSPA/HSP70 chaperones and associated co-chaperones, such as DNAJ/HSP40, which participates in the function of HSPA/HSP70, alone are insufficient to prevent the appearance of HSF1 trimers, suggesting that the acquisition of transcriptional activity is a separate process from that of the regulation of trimer formation (Figure 1.7) (Morimoto, 1998)

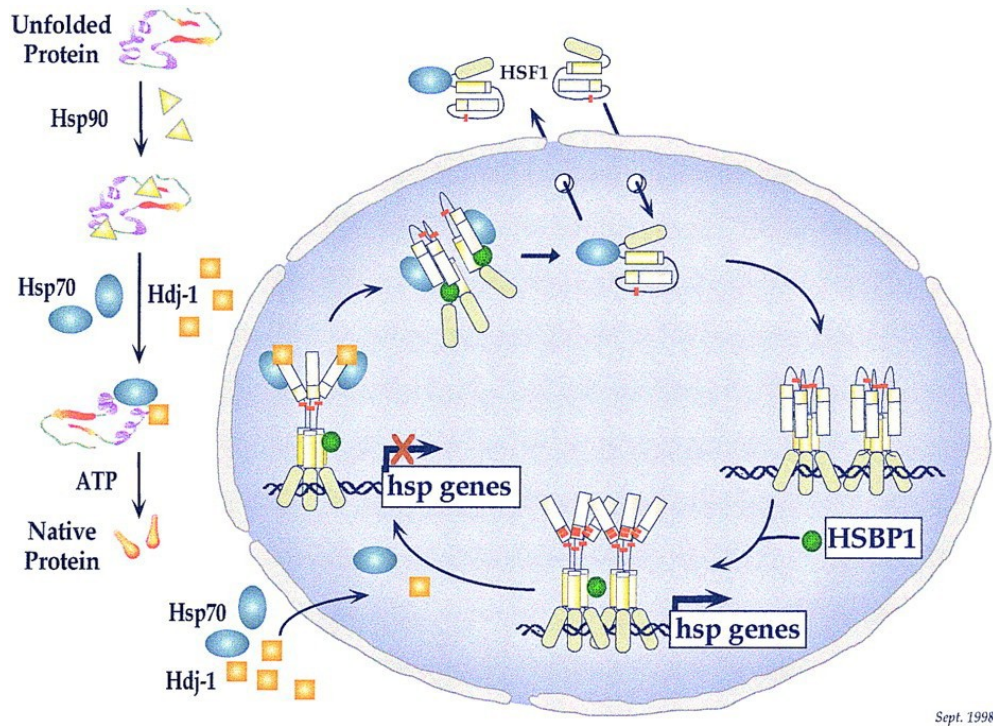


Figure 1.7. Regulation of the heat shock response and the HSF cycle. HSF1 exists in a controlled state as an inert monomer in either the cytoplasm or the nucleus through transient interactions with a number of chaperones such as HSPA/HSP70, HSPC/HSP90 and DNAJ/HDJ1. In response to heat shock the transcriptional activity of HSF1 is suppressed by direct binding of HSPA/HSP70 and HDJ1. The heat shock factor binding protein 1 (HSBP1), which is a conserved protein of 76 amino acids containing an extended hydrophobic heptad repeat interacts and binds to the hydrophobic heptad repeats of both HSF1 and HSPA/HSP70. These events lead to the appearance of HSF1 inert monomers and the dissociation of HSF1 trimers. (Taken from Morimoto, 1998).

1.2 Heat shock proteins

It is necessary to describe the main heat shock protein families found in eukaryotic cells. However, due to the diversity of the heat shock protein families, which cover approximately five hundred client proteins, this section will focus on the five heat shock proteins studied examples.

1.2.1 Heat Shock Protein 27 (HSPB)

The ubiquitous heat shock protein 27 (HSPB/HSP27) has a molecular weight of 27 KDa and is a member of the small heat shock protein family. HSPB/HSP27 is highly conserved amongst all species and has high homology with the eye lens α -crystallin proteins (Ciocca *et al*, 1993). α -crystallin proteins are water soluble structural proteins that are present in high concentrations in the cytoplasm of eye lens fibre cells (de Jong *et al*, 1989). α -crystallin proteins exhibit chaperone-like properties, which include the ability to prevent the acceleration of denatured proteins; an increase in cellular tolerance to stress and assist in the maintenance of lens transparency and prevention of cataracts (Augusteyn, 2004; de Jong *et al*, 1989). HSPB/HSP27 production is induced by stimuli similar to those of the larger molecular weight heat shock proteins (Garrido *et al*, 1997; Garrido *et al*, 2006; Parcellier *et al*, 2003). It is constitutively expressed in a variety of tissues and this expression is up-regulated under conditions of stress, including disease (Kappe *et al*, 2003).

HSPB/HSP27 contains a homologous and highly conserved amino acid sequence the α -crystallin domain at the C-terminus. These sequences consist of ~ 100 residues with a homology between 20-60 % and which forms β sheets which are necessary for the formation of stable dimers. It also possesses a less conserved proline/phenylalanine rich region containing one or two WF/EPF motifs, tryptophan, phenylalanine / glutamic

acid, proline, phenylalanine, at the NH₂-terminus which are essential for protection, maintenance of the oligomeric structure and chaperone activity of the protein (Kim *et al*, 1998; Theriault *et al*, 2004; von Montfort *et al*, 2001). HSPB/HSP27 acts as an ATP-independent molecular chaperone which inhibits protein aggregation and stabilises partially denatured proteins, which ensures protein refolding by the HSPA/HSP70 complex (Ehrnsperger *et al*, 1997; Parcellier *et al*, 2003). Also, HSPB/HSP27 assists in cytoprotection, by protecting cells against apoptosis under stress conditions (Charette *et al*, 2000; Pandley *et al*, 2000; Paul *et al*, 2002). Apoptosis is inhibited by interactions with the cellular death associated protein DAXX7, facilitating the activation of protein kinase B, AKT, (Rane *et al*, 2003) and blocking the formation of the apoptosome, a multisubunit protein complex involved in the activation of apoptosis, (Pandley *et al*, 2000; Paul *et al*, 2002). HSPB/HSP27 has been shown to interact with different cytoskeletal elements affecting actin polymerization (Ehrnsperger *et al*, 1997), to modulate intracellular reactive oxygen species content, to prevent apoptotic cell death activated by various stimuli including heat, serum deprivation, tumour necrosis factor alpha (TNF- α) and many commonly used anti-cancer drugs such as etoposide, doxorubicin, vincristine and cisplatin (Garrido, 2002; Huott *et al*, 1991; Oesterreich *et al*, 1996; Parcellier *et al*, 2003).

Studies have shown HSPB/HSP27 to be phosphorylated in response to a number of extracellular-derived signals such as TNF- α , thrombin, and also under conditions of stress arising from heat shock, oxidative stress and disease (Charette *et al*, 2000; Landry *et al*, 1992). Phosphorylation of HSPB/HSP27 occurs at three serine residues, Ser-15, Ser-78 and Ser-82 (Landry *et al*, 1992; Stokoe *et al*, 1992), and is activated by a number of protein kinases including MAPKAPK-2, MAPKAPK-3, PKAC α , p70S6k, PKD1,

AKT1 and PCK7 (Butt *et al*, 2001; Doppler *et al*, 2005; Landry *et al*, 1992; Rane *et al*, 2003; Stokoe *et al*, 1992).

Overexpression of *HSPB/HSP27* in disease states, such as cancer, facilitates the adaptation of cells to stressful conditions by assisting in the suppression of apoptosis, leading to a more aggressive disease phenotype (Aldrian *et al*, 2002). Therefore, overexpression of *HSPB/HSP27* correlates with poor patient prognosis in a variety of cancers such as include: colorectal, prostate, testis, breast, and ovarian cancers (Ciocca *et al*, 2005; Garrido *et al*, 1998; Garrido *et al*, 1997; Oesterreich *et al*, 1993; Richards *et al*, 1996). In *vivo*, *HSPB/HSP27* has also been implicated in cellular thermotolerance, although the expression of *HSPB/HSP27* alone is insufficient to induce this state and requires the assistance of higher molecular weight heat shock proteins (Trautinger *et al*, 1997).

1.2.2 Heat Shock Protein 40 (DNAJ)

The heat shock protein 40 (DNAJ/HSP40) family, with an average molecular weight of 40 KDa, is a large protein family consisting of over 100 members. Members of the DNAJ/HSP40 family have been classified into three subtypes in relation to their domain structures, (subfamily A, Type I; subfamily B, Type II; subfamily C, Type III) (Cheetham and Caplan, 1998; Ohtsuka and Hata, 2000; Uchiyama *et al*, 2006). All subfamilies contain a highly conserved J domain of ~ 78 residues (Fink, 1999; Laufen *et al*, 1998). In subfamilies I and II the J-domain is located at the N-terminus, whereas in subfamily III, the J-domain may be located at any position within the protein sequence. Both subfamilies I and II have a peptide-binding fragment located at the C-terminus of the proteins. The N-terminal J-domains are connected to the peptide-binding fragments via a Gly/Phe rich linker in subfamilies I and II. Subfamily I members, for example the human member HDJ2, contain two Zinc finger motifs between the J-domain and the C-terminal peptide-binding fragment. In contrast, subfamily II members, such as the human member HDJ1, lack this feature (Caplan *et al*, 1992; Cyr *et al*, 1994; Zhong and Arndt, 1993). Subfamily III members, as mentioned above, contain the J-domain, but this may be located at any position within the protein and therefore lack the other conserved domains found in subfamily I and II members.

The main role of DNAJ/HSP40 is that of a cochaperone, which specifies the cellular action of HSPA/HSP70 proteins. DNAJ/HSP40 plays an important role in protein folding, unfolding, translation, translocation and degradation, primarily by stimulating the ATPase activity of HSPA/HSP70 proteins (Fink *et al*, 1999, Minami *et al*, 1996; Qiu *et al*, 2006). DNAJ/HSP40 in both eukaryotic and prokaryotic cells interacts with HSPA/HSP70 in the presence of ATP suppressing protein aggregation (Cyr, 1994; Fink *et al*, 1999). It has been suggested that DNAJ/HSP40 binds to the non-native polypeptide first and then delivers the non-native polypeptide to HSPA/HSP70 for

folding (Bukau and Horwich, 1998; Gething and Sambrook, 1992; Hartl, 1996). The main function of DNAJ/HSP40 is to regulate adenosine triphosphate (ATP) dependent polypeptide binding by HSPA/HSP70 protein (Fan *et al*, 2003; Hartl and Hayer-Hartl, 2009; Szabo *et al*, 1994). DNAJ/HSP40 strongly accelerates the hydrolysis of ATP to adenosine diphosphate (ADP) resulting in the closing of the α -helical lid of the peptide binding domain and tight binding of the peptide substrate by HSPA/HSP70. DNAJ/HSP40 dissociates from HSPA/HSP70 (Hartl and Hayer-Hartl, 2009). Interaction of the substrate with HSPA/HSP70 is mediated by the J domain, which is present in all DnaJ/HSP40s (Hartl and Hayer-Hartl, 2009; Mayer *et al*, 2000). DNAJ/HSP40 also directly interacts with unfolded polypeptides and can convert HSPA/HSP70 to protein substrates (Hartl and Hayer-Hartl, 2009; Young *et al*, 2003). Following ATP-hydrolysis, a number of nucleotide exchange factors (NEF), such as Bag, and HSPBP1, bind to the HSPA/HSP70 ATPase domain and catalyse ADP-ATP exchange, which results in the opening of the α -helical lid and release of the substrate (Hartl and Hayer-Hartl, 2009). The released substrate either folds to a native state; or is transferred to downstream chaperones, such as HSPC/HSP90, or rebinds to HSPA/HSP70 (Hartl and Hayer-Hartl, 2009).

The J domain, which is common to all DNAJ/HSP40 family members, is responsible for the regulation of HSPA/HSP70 ATPase activity. Interactions of HSPA/HSP70 proteins with DNAJ/HSP40 proteins produce HSPA/HSP70-DNAJ/HSP40 heterodimers which assist certain processes at specific locations within a cell (Fan *et al*, 2003; Liu *et al*, 1998). DNAJ/HSP40 also functions as homodimers which interact with non-native polypeptides of at least six residues in length, through hydrophobic interactions (Cyr, 1994; Fan *et al*, 2003; Fink *et al*, 1999; Li *et al*, 2009). The peptide-binding site of DNAJ/HSP40 also serves as the binding site for the C-terminal EEVD motif of

HSPA/HSP70 through charge-charge interactions (Freeman *et al*, 1995; Li *et al*, 2009; Qian *et al*, 2002).

The overexpression of *DNAJ/HSP40* and *HSPA/HSP70* has been observed in colorectal (Kanazawa *et al*, 2003) and gastric tumour tissues (Isomoto *et al*, 2003). However, immunohistochemical analysis has shown only a small proportion of samples which were positive for *DNAJ/HSP40* in gastric cancer tissues (Isomoto *et al*, 2003). *HSPA/HSP70* and *DNAJ/HSP40* were shown to be down-regulated in undifferentiated cancers, suggesting that HSPA/HSP70-DNAJ/HSP40 chaperone systems could have a role in tumour differentiation (Isomoto *et al*, 2003; Kanazawa *et al*, 2003).

1.2.3 Heat Shock Protein 60 (HSPD)

The heat shock protein 60 family (HSPD/HSP60) is named after the average molecular weight of its members of 60 KDa. Their genes are either moderately stress inducible or are highly expressed constitutively (Jindal *et al*, 1989; Neuer *et al*, 2000). Although the majority of HSPD/HSP60 is located in the mitochondria, ~ 15 -20% of cellular HSPD/HSP60 is located in extramitochondrial sites, such as the cytosol, peroxisomes, endoplasmic reticulum and at the cell surface (Gupta *et al*, 2002; Pfister *et al*, 2005; Stefano *et al*, 2009; Stetler *et al*, 2010). They are responsible for the synthesis, transportation and refolding of essential proteins from the cytoplasm into the mitochondrial domain. Their main function is the ATP-dependant folding and/or refolding of approximately 15-30% of total cellular proteins and they also assist in the folding of linear amino acid chains into their respective tertiary structures (Ranford *et al*, 2000; Trivedi *et al*, 2010; Urushibara *et al*, 2007).

HSPD/HSP60 has two main roles in relation to mitochondrial protein transport: the catalysis of the folding of proteins destined for the mitochondrial matrix and maintenance of proteins in an unfolded state for transmembrane transportation (Koll *et al*, 1992). *In vitro* HSPD/HSP60 has been identified as a single-stranded DNA (ssDNA) binding protein that simulates DNA replication (Smiley *et al*, 1992). HSPD/HSP60 has been found to associate with ssDNA regions and to bind to ssDNA of active sequences with high specificity for the template of a putative origin of mitochondrial DNA (mtDNA) replication in a strand specific manner (Kaufman *et al*, 2003; Kaufman *et al*, 2000).

Under normal physiological conditions, HSPD/HSP60 is located typically in the mitochondria, but can also be found in the cytoplasm (Itoh *et al*, 2002; Jindal *et al*, 1989; Neuer *et al*, 2000). HSPD/HSP60 is composed of monomers that form a double heptameric ring structure (Cheng *et al*, 1990) comprised of a large central cavity whereby unfolded proteins bind via hydrophobic interactions (Fenton *et al*, 1994). Each subunit of HSPD/HSP60 contains three domains: the apical domain, the equatorial domain, which contains the binding site for ATP, and the intermediate domain which links the apical and equatorial domains (Ranford *et al*, 2000).

HSPD/HSP60 proteins play an important role in preventing apoptosis in the cytoplasm and are also associated with the immune response and cancer (Hansen *et al*, 2003; Itoh *et al*, 2002). The cytoplasmic HSPD/HSP60 forms a complex with various other proteins that are responsible for apoptosis and regulates their activity (Itoh *et al*, 2002). HSPD/HSP60 has been shown to influence apoptosis in the immune response (Hansen *et al*, 2003) and in cancer cells (Itoh *et al*, 2002).

In cancer, *HSPD/HSP60* expression appears either to inhibit apoptotic and necrotic cell death or plays a role in the activation of apoptosis, dependent on the tumour type (Capello *et al*, 2006; Urushibara *et al*, 2007). A loss of *HSPD/HSP60* expression in bladder carcinomas, –indicates a poor prognosis and the risk of developing tumour infiltration, (Lebret *et al*, 2003). In the initial stages of ovarian cancer, *HSPD/HSP60* is over-expressed with a decrease in expression as the disease progresses, associated also with tumour aggressiveness (Lebret *et al*, 2003; Schnieder *et al*, 1999). These changes in *HSPD/HSP60* expression levels have the potential to be –new useful biomarkers for diagnostic and prognostic purposes (Capello *et al*, 2006).

1.2.4 Heat Shock Protein 90 (HSPC)

The heat shock protein 90 family (HSPC/HSP90) has an average molecular weight of 90 KDa and is one of the most abundant of the heat shock proteins, comprising approximately 1 - 2% of total cellular proteins under unstressed conditions (Csermely *et al*, 1998). HSPC/HSP90 plays an important role in the folding of newly synthesized proteins and the stabilization and refolding of denatured proteins after stress conditions; intracellular protein transport; protein degradation; facilitation of cell signalling and in suppressing the aggregation of numerous client/substrate proteins (Buchner, 1999; Jakob *et al*, 1995; Miyata and Yahara, 1992; Wiech *et al*, 1992).

The HSPC/HSP90 family consists of both inducible and constitutive isoforms, with the two major cytoplasmic isoforms being HSPC1a/HSP90 α (the inducible isoform and HSPC2a/HSP90 β (the constitutive isoform) (Chen *et al*, 2006; Csermely *et al*, 1998). A further identified isoform of the HSPC/HSP90 family is HSPC3a/HSP90N, a constitutive protein associated with cellular transformation (Grammatikakis *et al*, 2002; Sreedhar *et al*, 2004). A number of HSPC/HSP90 analogues have also been reported: HSPA9/Grp75, localized in the endoplasmic reticulum (ER) and HAPC5a/HSP75/TRAP1, localized in the mitochondrial matrix (Csermely *et al*, 1998; Sreedhar *et al*, 2004).

All members of the HSPC/HSP90 family contain three structural domains: a highly conserved N-terminal nucleotide binding pocket ~ 25 KDa in length which possesses a high affinity ATP binding site (Chadli *et al*, 2000; Pearl and Prodromou, 2000; Prodromou *et al*, 1997; Prodromou and Pearl, 2003), a central domain ~ 40 KDa in length which is involved in substrate protein folding and ATPase activity

(Grammatikakis *et al*, 2002; Meyer *et al*, 2003) and a C-terminal domain ~ 12 KDa in length, which acts as a second ATP binding site when the N-terminal domain is occupied (Garnier *et al*, 2002; Marcu *et al*, 2000; Soti *et al*, 2002).

HSPC/HSP90 expression can be associated with various types of cancers. *HSPC/HSP90* in cancer cells exists in a -hyperactive state in the diseased tissue compared to that of the normal surrounding tissue (Kamel *et al*, 2003). In this highly active state *HSPC/HSP90* influences various other proteins that contribute to the pathogenesis of the disease. These include cell growth promotion, evasion of apoptosis and stimulation of angiogenesis (Kamel *et al*, 2003). Over-expression of *HSPC1a/HSP90α* has been associated with poor prognosis of leukaemia (Yufu *et al*, 1992), pancreatic carcinoma (Gress *et al*, 1994) and breast cancer (Jameel *et al*, 1992).

A study undertaken by Ogata *et al*, (2000) in pancreatic carcinoma tissue showed over-expression of *HSPC1a/HSP90α* compared to pancreatitis and normal pancreas tissue. In contrast, *HSPC2a/HSP90β* was constitutively over-expressed in pancreatic carcinoma, pancreatitis and normal pancreatic tissues. These results suggest that *HSPC2a/HSP90β* is correlated to structural conformation by forming complexes with tubulin and actin which constitute the cytoskeleton, and that *HSPC1a/HSP90α* is directly or indirectly involved with cell proliferation and carcinogenesis (Ogata *et al*, 2000).

1.2.5 Heat Shock Protein 70 (HSPA)

The heat shock protein 70 (HSPA/HSP70) family is the most conserved group of the heat shock proteins (Oehler *et al*, 2000). HSPA/HSP70 is an anti-apoptotic protein which blocks a number of steps in the stress-induced apoptotic pathway (Kang *et al*, 2009; Lanneau *et al*, 2007). Its functions include the folding and assembly of newly synthesized proteins, refolding of misfolded and aggregated proteins, regulation of apoptosis, membrane translocation of organellar and secretory proteins, and controlling the activity of regulatory proteins (Figure 1.8) (Beckman *et al*, 1990; Kang *et al*, 2009; Mayer and Bukau, 2005; Seidberg *et al*, 2003).

During stress conditions, HSPA/HSP70 is induced at high levels through a combination of transcriptional activation, preferential translation and mRNA stabilization. It inhibits cell death by preventing the aggregation of cell proteins (Calderwood *et al*, 2005; Lindquist and Craig, 1988). HSPA/HSP70 proteins are weak ATPases which cycle through high and low affinity substrate binding states by nucleotide hydrolysis. An ATP bound HSPA will bind substrates with low affinity, whereas an adenosine 5' diphosphate (ADP) bound HSPA will bind substrates with high affinity (Schmid *et al*, 1994; Shaner and Morano, 2007).

An extensive literature and database search undertaken by Brocchieri *et al*, (2008) identified 130 human HSPA/HSP70 proteins. Subsequent analysis revealed 86 groups of non-identical sequences which were further analysed and clustered into 13 groups. A representative sequence was selected from each of the 13 groups to query the human genome using NCBI Build 36.1 and to identify conserved and diverged sequences. This process yielded 47 loci, which included coding HSPA/HSP70-like sequences, of which

seventeen corresponded to known genes recognized in the genome annotation (Table 1.4). Included within the latter seventeen was *HSPA 7*, which is considered to be a pseudogene.

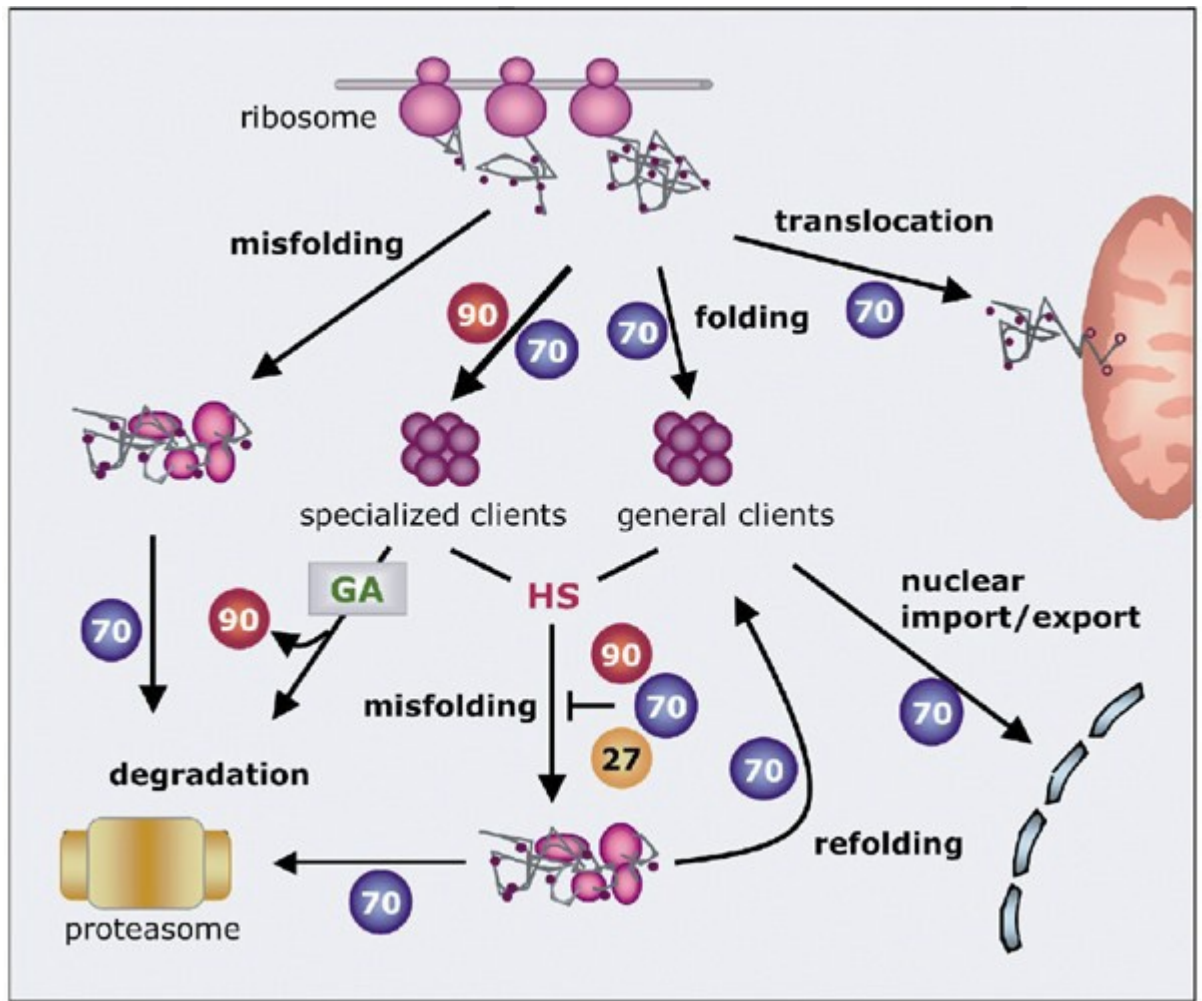


Figure 1.8 Chaperone-assisted protein folding (taken from Mosser and Morimoto, 2004). HSPA/HSP70 is required for the productive folding of newly synthesized proteins, translocation across intracellular membranes and import into and export of the nucleus. Heat shock and other protein damaging stresses cause protein misfolding and aggregation, which can be limited by heat shock proteins. These misfolded proteins can be rescued by the folding activity of HSPA/HSP70. Proteins that cannot be refolded are targeted to the proteasome for degradation.

Table 1.4. Expression and location of the human HSPA genes (Taken from Brocchieri *et al*, 2008)

| Gene | Cell Locale | Tissue | Expression | Location |
|----------------|--|--|---|-----------------|
| <i>HSPA8</i> | Cytosol | All tissues highly expressed | Constitutive. Moderately induced by heat shock. | 11q24.1 |
| <i>HSPA12A</i> | NK | Brain/kidney/muscle | Constitutive. Induced by stress. | 10q25.3 |
| <i>HSPA12B</i> | NK | Skeletal/heart muscle | Constitutive. Induced by stress. | 20p13 |
| <i>HSPA9B</i> | Mitochondria/ER/Cytosol/cystolic vesicles/membrane surface | Many tissues | Constitutive | 5q31.2 |
| <i>HSPA4</i> | Cytosolic | Most tissues | Constitutive. Not induced by heat shock. | 5q31.1 |
| <i>HSPA4L</i> | Cytosol | Testis | Constitutive. Induced by heat shock. | 4q28.1 |
| <i>HSPH1</i> | Cytosol | Most tissues | Constitutive. Induced by heat shock. | 13q12.3 |
| <i>HYOU1</i> | ER/Cytosol | Liver/pancreas | Induced by ER stressors | 11q23.3 |
| <i>HSPA14</i> | Cytosol, associated ribosomes | Many tissues | Undetermined | 10p13 |
| <i>STCH</i> | Microsomas | All tissues | Constitutive | 21q11.2 |
| <i>HSPA2</i> | Nucleus | Testis/skeletal and heart muscles/oesophagus/brain | Constitutive | 14q23.3 |
| <i>HSPA1A</i> | Cytosol | All tissue | Strongly induced by heat shock | 6p21.33 |
| <i>HSPA1B</i> | Cytosol | All tissue | Strongly induced by heat shock | 6p21.32 |
| <i>HSPA1L</i> | Cytosol | Spermatides | Constitutive. Not induced by heat shock. | 6p21.33 |
| <i>HSPA6</i> | Cytosol/Nucleus | Most tissues | Induced by heat shock | 1q23.3 |
| <i>HSPA7</i> | | | | 1q23.3 |
| <i>HSPA5</i> | ER | All tissue | Induced by ER stressors | 9q33.3 |

The human *HSPA* genes encode proteins which have been clustered into seven major distinct evolutionary groups (Figure 1.9), with noticeable subgroups relating to phylogenetic and other related data, including pseudogenes, exon-intron and protein features (Brocchieri *et al*, 2008).

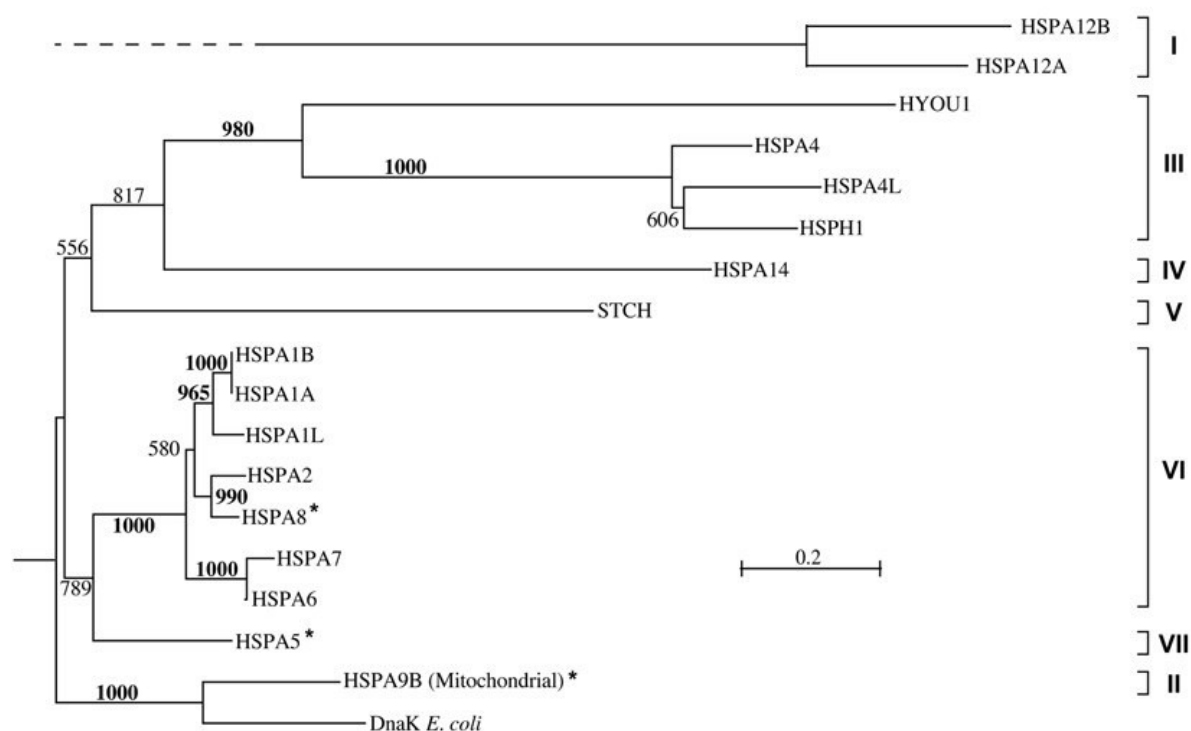


Figure 1.9. Phylogenetic tree of 17 human HSPA proteins. This is based on the alignment of their protein products defined by bootstrap support values over 85 %. Group I refers to HSPA12B and HSPA12A, Group II, HSPA9B AND DnaK, Group III, HYOU1, HSPA4, HSPA4L and HSPH1, Group IV, HSPA14, Group V, STCH, Group VI, HSPA1B, HSPA1A, HSPA1L, HSPA2, HSPA8, HSPA7 and HSPA6 and Group VII, HSPA5. The distance scale of 0.2 = 20% represents the differences between sequences. The branch lengths are proportional to the scale, so two sequences with longer branches are more diverged than sequences with shorter branches (taken from Brocchieri *et al*, 2008).

Group I is characterised by two similar, but diverged, sequences, HSPA12A and HSPA12B. Both sequences possess identical exon-intron structures and 12 exons have been located within their coding regions. Group II includes the mitochondrial protein HSPA9B, encoded by a nuclear gene which possesses 17 exons, and the DnaK sequence from *E. coli*. Group III proteins include three closely related 105/110 KDa proteins whose genes all have a common structure of 18 – 19 exons. These Group III proteins include HSPA4L, HSPA4 and HSPH1, which all occur as two isoforms. Also contained within Group III is the distantly related sequence HYOU1, which encodes the 170 KDa protein, Grp170, known to exist in three isoforms, and whose genes possess 16, 24 or 25 exons respectively (Brocchieri *et al*, 2008).

Group IV includes the sole sequence HSPA14, of which two isoforms exist, possessing 4 and 14 exons respectively. Although closely associated with Group III, cluster analysis showed a bootstrap support value of 81% for HSPA14, which is below the bootstrap threshold value of 85% and therefore the latter protein is allocated a separate evolutionary group. Group V also comprises a single sequence, STCH, which has 5 exons within the coding region and is weakly related to Group III and Group IV, having a bootstrap support value of 55.6% (Brocchieri *et al*, 2008).

Group VI contains seven sequences, each with bootstrap values of 100%, which have been divided into three distinct subgroups. Subgroup one includes the intronless sequences for HSPA1A, HSPA1B and HSPA1L, which are positioned within the MHC-III region on the short arm of chromosome 6. Subgroup two contains HSPA2 and HSPA8, existing as two isoforms which possess 7 – 8 exons, whilst subgroup three comprises HSPA6 and HSPA7. Finally, Group VII contains one sequence HSPA5,

which has 8 exons within the coding region and is closely related to Group VI having a bootstrap support value of 78.9% (Brocchieri *et al*, 2008).

The HSPA family share significant similarities in sequence and structure, based on highly conserved bipartite domain structure (Shaner and Morano, 2007). This structure comprises three major functional domains, including a highly conserved N-terminal ATPase domain (NBD) with a molecular mass of ~ 44 KDa which controls the closing and opening of the peptide domain. An adjacent well-conserved substrate binding domain (SBD), with a molecular mass of ~ 18kD, contains a hydrophobic pocket and lid-like structure over the pocket and a conserved C-terminal domain, with a molecular mass of ~ 10 KDa, which chaperones denatured proteins and peptides (Fiege and Polla, 1994; Hartl, 1996; Hartl and Hayer-Hartl, 2002; Mao *et al*, 2006; Mayer and Bukau, 2005; Mosser and Morimoto, 2004; Osipiuk *et al*, 1999; Wisniewska *et al*, 2010). When non-active, HSPA/HSP70 is usually in an ATP bound state. As newly synthesized proteins emerge, the SBD of HSPA/HSP70 recognizes and interacts with sequences of hydrophobic amino acid residues, stimulating the ATPase activity of HSPA/HSP70 and increasing ATP hydrolysis. As ATP is hydrolyzed to ADP, the hydrophobic pocket tightly closes, binding the peptides to the SBD (Hartl, 1996; Hartl and Hayer-Hartl, 2002; Mao *et al*, 2006; Mayer and Bukau, 2004; Mosser and Morimoto, 2004). In the case of partially synthesized peptide sequences, HSPA/HSP70 will prevent aggregation, allowing them to re-fold correctly. All three domains play important roles in tumour immunity, not only by enabling intracellular HSPA/HSP70 to prevent tumour apoptosis, but also to promote the generation of stable complexes with cytoplasmic tumour antigens stimulating anti-tumour immunity (Calderwood *et al*, 2005; Schmitt *et al*, 2007). Under normal conditions, HSPA/HSP70 functions as an ATP dependent molecular chaperone and is found mainly in the cytosol. Various stress

conditions such as heat shock, hypoxia and cancer increase the synthesis of HSPA/HSP70, and also cause it to migrate to the nucleus and associate with nuclear proteins (Santoro, 2000).

Heat shock proteins can be toxic if present in cells for any prolonged period of time and must therefore be stringently regulated. Theodorakis *et al*, (1999) examined the expression of *HSPA/HSP70* after heat shock in thermotolerant (heat resistant) and non-thermotolerant human hepatoblastoma cells. During and after heat shock, *HSPA/HSP70* transcriptional activity in both thermotolerant and non-thermotolerant cells was very similar. mRNA stability and expression of *HSPA/HSP70* was reduced in thermotolerant cells, compared to the non-thermotolerant cells after heat shock, suggesting that the thermotolerant cells possibly limit *HSPA/HSP70* expression to avoid cytotoxic effects.

The *HSPA/HSP70* gene family is of particular interest with regard to three members (*HSPA1A/HSP70-1*, *HSPA1B/HSP70-2* and *HSPA1L/HSP70-Hom*; Milner and Campbell, 1990). The nomenclature now used is specific to the allelic level of the genes, whose genes are located within the major histocompatibility complex class III region (MHC-III) on the short arm of chromosome 6 (6p21.3), and are intronless (Figure 1.10).

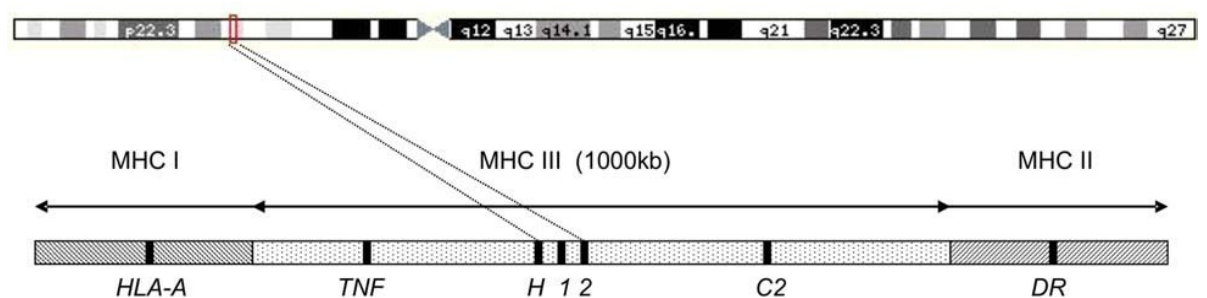


Figure 1.10. Localization of the three HSPA genes (H = HSPA1L, 1 = HSPA1A, 2 = HSPA1B). *HSPA1A* and *HSPA1B* are 11 kb apart and *HSPA1L* is 4 kb telomeric to *HSPA1A*. (Taken from Singh *et al*, 2007)

Although these three genes have similar sequences, they differ in their regulation. Both *HSPA1A* and *HSPA1B* have been shown to encode identical protein products of 641 amino acids, and *HSPAIL* has been identified at a location 4 kb telomeric to *HSPA1A* with a 90% similarity in amino acid sequence (Sargent *et al*, 1989a, Sargent *et al*, 1989, Milner and Campbell, 1990). Both *HSPA1A* and *HSPA1B* have been shown to be highly expressed in mammalian cells that have been subjected to heat shock at 42°C. *HSPA1A* is also constitutively expressed at low levels. *HSPAIL*, which has no heat shock consensus sequence, has been shown to be expressed at low levels both constitutively and after heat shock at 42°C, with no comparable difference in levels (Milner and Campbell, 1990).

1.2.5.1. Polymorphisms in HSP70 genes

The term polymorphism relates to the presence of multiple allelic variants of a gene and is a significant part of the genetic variation that exists in a population. The MHC region is one of the most highly polymorphic regions in the human genome. Single nucleotide polymorphisms (SNPs) are the most common type of genetic variation within the human genome. They involve variation between two sequences by a single base pair, often due to a base-pair substitution, of which two subtypes exist: transitions, whereby a purine or pyrimidine base is substituted by another and transversions, whereby a purine is substituted by a pyrimidine or vice versa (Singh *et al*, 2007).

Four different polymorphic locations have been described in *HSPA1A* (Table 1.5). Three lie within the 5' flanking region of the gene, which is not transcribed into RNA and the 5' untranslated region (UTR). They are located at positions -110 (A to C transversion), 120 (T to C transition) and 190 (G to C transversion). The fourth

polymorphic location is found in the coding region of the gene at the position 438 (C to T transition) (Singh *et al*, 2007).

The *HSPA1B* gene also has four different polymorphic locations (Table 1.5). One lies within the 5' flanking region of the gene at the position 145 (C to T transition), and two lie within the coding region of the gene at positions 1267 (A to G transition) and 2074 (C to G transversion). The fourth polymorphism is located in the 3' UTR region at the position 2257 and is a result of a duplication of the pentameric sequence AAGTT which gives rise to two alleles (183bp and 188bp) differing in 5bp (Singh *et al*, 2007).

Four different polymorphic sites have also been discovered in *HSPA1L* (Table 1.5). These lie within the coding region of the gene at positions 1097 (C to T transition), 2180 (G to A transition), 2437 (C to G transition) and 2763 (G to A transition). The polymorphisms at position 2437 and 2763 lead to amino acid substitutions at position 493 from Methionine (Met) to Threonine (Thr) and position 602 from Glutamic acid (Glu) to Lysine (Lys) respectively (Singh *et al*, 2007).

Table 1.5. Single nucleotide polymorphisms in three human HSPA genes (taken from Singh *et al*, 2007)

| SNP position | Nucleotide Change | |
|----------------------|---|---|
| <i>HSPA1A</i> | | |
| -110 (5' flanking) | A to C transversion | |
| 120 (5' UTR) | T to C transition | |
| 190 (5' UTR) | G to C transversion | |
| 438 (coding) | C to T transition | |
| <i>HSPA1B</i> | | |
| 145 (5' flanking) | C to T transition | |
| 1267 (coding) | A to G transition | Generates a <i>Pst I</i> site giving rise to two alleles, L allele (8.5 kb) and U allele (9.0 Kb) identified according to their length |
| 2074 (coding) | C to G transition | |
| 2257 (3'UTR) | Penta-duplication of the sequence AAGTT | This gives rise to two alleles A1 (183 bp) and A2 (188 bp) differing in 5 bp. |
| <i>HSPA1L</i> | | |
| 1097 (coding) | C to T transition | |
| 2180 (coding) | G to A transition | |
| 2437 (coding) | C to T transition (aa substitution) | An AA change at position 493 from a non-polar hydrophobic Methionine (Met) to a polar neutral Threonine (Thr). The change of AA could be associated with altered peptide binding specificity and efficiency of HSP70. |
| 2763 (coding) | G to A transition (aa substitution) | An AA change at position 602 from Glutamic acid (Glu) to Lysine (Lys). This change lies within the <i>Nco I</i> restriction site |

Due to the diversity of heat shock protein families, which cover approximately five hundred client proteins, the present study will only focus on HSPA/HSP70.

1.3 Activation of HSPA/HSP70

1.3.1 Heat shock

As stated previously, heat shock proteins are up-regulated in response to a variety of stressful conditions, including thermal stress (Tolson and Roberts, 2005; Welch, 1993). Environmental temperatures have an impact on the cellular stress response and determine how cells adapt to subsequent changes in temperature. This is referred to as thermotolerance, a cellular adaptation resulting from a single severe non-lethal exposure to heat which allows cells to better survive subsequent potentially lethal heat stress episodes. The accumulation of stress-induced heat shock proteins in cells is associated with thermotolerance and is responsible for their ability to survive not only lethal heat stress, but also a variety of other stresses. The characteristics for thermotolerance are dependant on survival of the cell(s) exposed to an otherwise lethal heat stress, synthesis of heat shock proteins and a short duration of the thermotolerant state (Moseley, 1997).

As discussed previously, Theodorakis *et al*, (1999) compared the expression and mRNA degradation in thermotolerant (cells that had been subjected to an initial heat shock) and non-thermotolerant (cells under normal conditions that had not been subjected to an initial heat shock) human liver hepatocellular carcinoma (HepG2) cells after heat shock. The normal physiological temperature for human cells is 37°C. Induction of heat shock protein genes occurs when heat shock is applied just above the normal growth temperature. Cells were made thermotolerant by the application of a nonlethal heat shock (43°C for 1.5 h) followed by a recovery period of 24 h at 37°C. Both thermotolerant and non-thermotolerant cells were thermally stressed at 43°C for 1.5 h, followed by a 6 h recovery period at 37°C. Results showed limited expression of

HSPA/HSP70 in thermotolerant cells, compared with non-thermotolerant cells after heat shock. It was suggested that this was due to both a decrease in *HSPA/HSP70* transcription and an increase in *HSPA/HSP70* mRNA degradation, thus increasing cellular levels of this protein in the recovery period, during which cells returned to non-heat shock conditions 37°C (Theodorakis *et al*, 1999). In contrast, during the heat shock period, no significant differences were observed in the levels of *HSPA/HSP70* transcription in thermotolerant and non-thermotolerant cells. *HSPA/HSP70* mRNA was observed to be less stable in thermotolerant cells, with a half-life in non-thermotolerant cells of approximately 1 h and approximately half an hour in thermotolerant cells. However, the half-life of *HSPA/HSP70* has previously been reported to be longer in comparison to the recovery period after stress (Theodorakis and Morimoto, 1987). The explanation suggested for this is that, under normal conditions, levels of *HSPA/HSP70* mRNA are unstable, but during heat shock the mRNA becomes more stable because its degradation is affected by stress. In thermotolerant cells subjected to heat shock, mRNA degradation begins immediately after returning to normal conditions, and therefore it is suggested that mRNA degradation is accelerated in thermotolerant cells resulting in a reduced *HSPA/HSP70* mRNA half-life.

1.3.1 Glioma

Gliomas, in the form of astrocytomas, anaplastic astrocytomas and glioblastomas, are the most common primary brain tumours and comprise about 2% of all newly diagnosed cancers every year in the UK (Chandana *et al*, 2008; Khalil, 2007). Gliomas are defined as tumours of the central nervous system (CNS), the majority of which arise from glial cells displaying histological, immunohistochemical and ultrastructural evidence of glial differentiation (Khalil, 2007). The classification of brain tumours is based upon cell morphology and the degree of malignant behaviour, which is in turn dependent on nuclear atypia, mitoses, microvascular proliferation and necrosis (Behin *et al*, 2003). The World Health Organization (WHO) classifies brain tumours into various subtypes such as astrocytoma, oligodendroglioma, oligoastrocytoma and ependymoma and into four malignancy grades; Grade I pilocytic astrocytoma, Grade II diffuse astrocytoma (including oligodendroglioma), Grade III anaplastic astrocytomas and Grade IV glioblastoma (Table 1.6) (Khalil, 2007; Kleihues *et al*, 2002; Louis *et al*, 2007).

Table 1.6. WHO grading and survival of gliomas (adapted in part from Christine *et al*, 2005).

| WHO Grade | Glioma | Histological Features | Survival |
|-----------|--|---|-------------|
| I | Pilocytic astrocytoma | Rosenthal fibres | +10 years |
| II | Diffuse astrocytoma/ oligodendroglioma/ mixed oligoastrocytoma | Moderate hypercellularity with occasional nuclear atypia | +5 years |
| III | Anaplastic astrocytoma / oligodendroglioma/ oligoastrocytoma | Increased hypercellularity, Nuclear atypia with high mitotic activity, microvascular proliferation | 2-3 years |
| IV | Glioblastoma multiforme | Marked nuclear atypia, mitotic activity, prominent endothelial proliferation and tumour necrosis | 9-12 months |

Pilocytic astrocytomas are defined as Grade I tumours and characterised by their slow growth rate and regressive nature and are often considered as benign tumours. Pilocytic astrocytomas generally develop in young children and young adults (Behin *et al*, 2003; Parsa and Givard, 2008). They usually originate in the optic tracts, hypothalamus or basal ganglia and in the cerebellum and brainstem (posterior fossa) but they may occur in any area where astrocytes are present, such as the spinal cord and the cerebral hemispheres (Behin *et al*, 2003; Kleihues & Cavenee, 2000). Imaging using MRI and CT scans show pilocytic astrocytomas as solid, cystic, well circumscribed and contrast-enhancing tumours (Figure 1.11) (Behin *et al*, 2003).

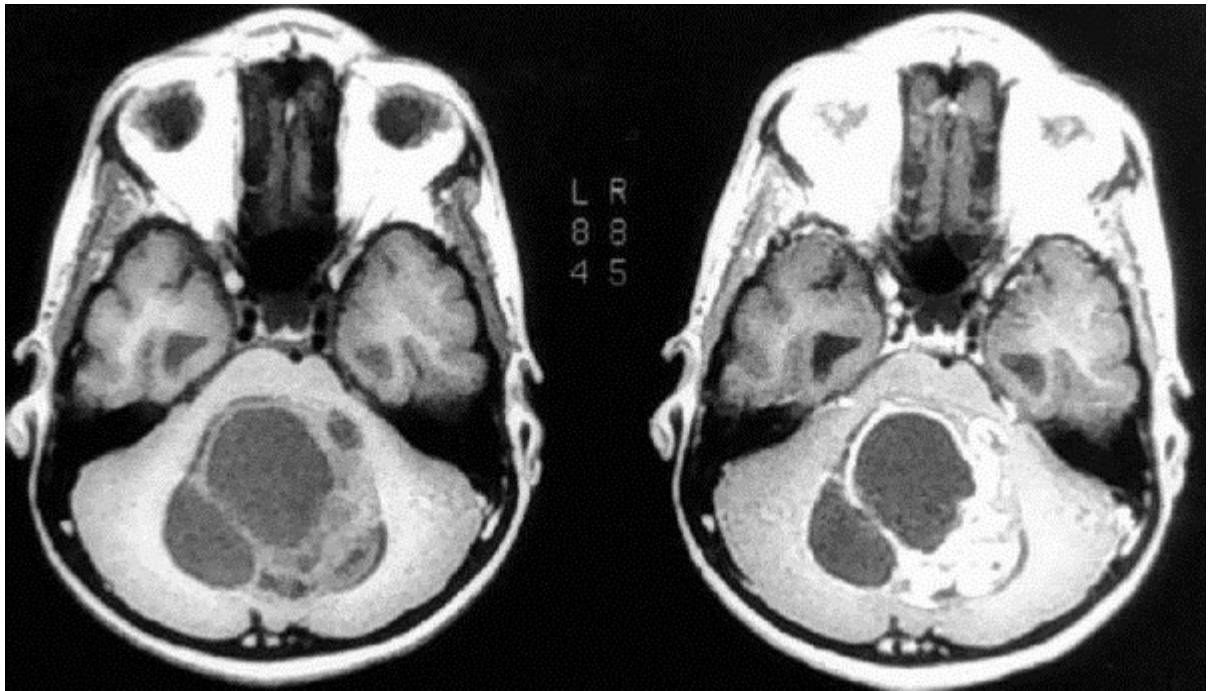


Figure 1.11. Magnetic resonance imaging showing features of pilocytic astrocytomas (rad.usuhs.mil/rad/who/JPA.html). Post-contrast image reveals a large irregular, multilobulated shape, in the enhancing mass in the cerebellar hemisphere.

Diffuse astrocytomas, referred to as Grade II tumours, such as oligodendrogliomas, are well differentiated tumours that are most prevalent in young adults, commonly located in the frontal, temporal and insular regions within the cerebral hemispheres (Behin *et al*, 2003; Huang *et al*, 2000). They originate from oligodendrocytes, which are the myelin-forming cells of the central nervous system, and which play a crucial role in the normal functioning of the brain (Jiang *et al*, 2007). Although slow growing, they have the potential for malignant progression and to develop into anaplastic astrocytomas (Grade III tumours) and eventually into glioblastomas (Grade IV tumours) (Behin *et al*, 2003; Huang *et al*, 2000). Contrast enhancement, whereby the contrast between normal and cancerous tissue is enhanced, is generally absent in MRI and CT images (Figure 1.12). However, presence of contrast enhancement would suggest malignant transformation into a higher grade of tumour (Behin *et al*, 2003).

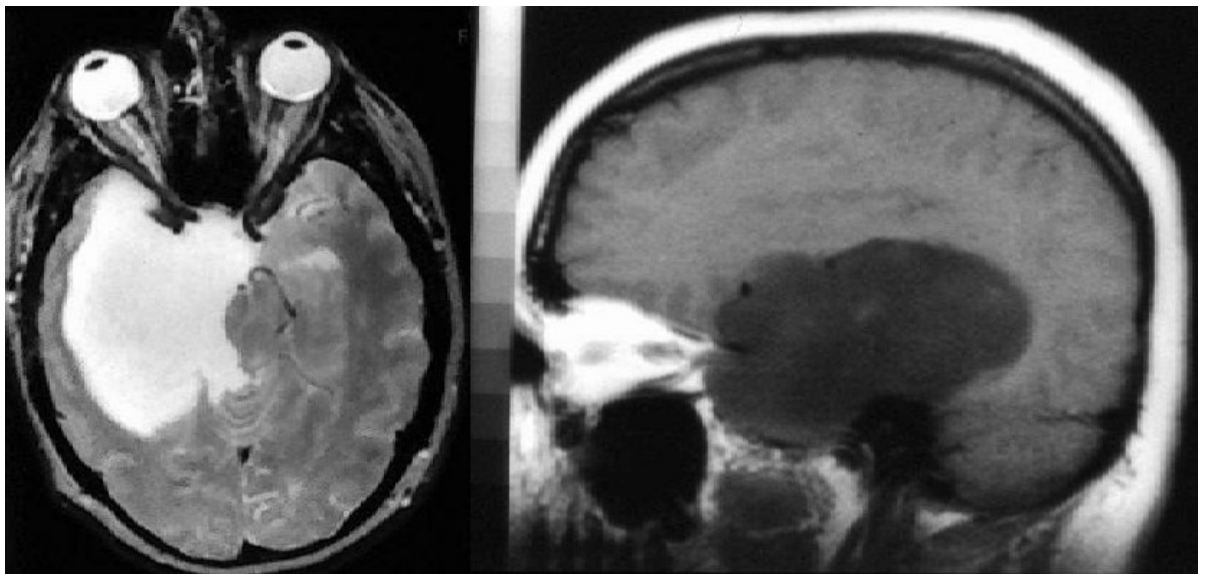


Figure 1.12. Magnetic resonance imaging showing features of a diffuse astrocytomas (rad.usuhs.mil/rad/who/JPA.html) post-contrast image reveals a large enhancing irregular, mass in the left frontal lobe.

Anaplastic astrocytomas, defined as Grade III tumours, may originate either *–de novo* without previous indication of a malignant lesion or as a transition from low grade lesions (Kleihues & Cavenee, 2000). The latter types of tumours are less common and generally represent a short-term intermediate lesion during malignant transition from Grade II to Grade IV tumour status (Behin *et al*, 2003; Huang *et al*, 2000). Histological astrocyte and anaplastic features include pleomorphism, increased tumour cellularity, high mitotic activity, microvascular activity, nuclear atypia and necrosis (Brat *et al*, 2008). On MRI and CT images (Figure 1.13) they present as an irregular hypodense lesion with varying degrees of contrast enhancement and oedema (Behin *et al*, 2003).

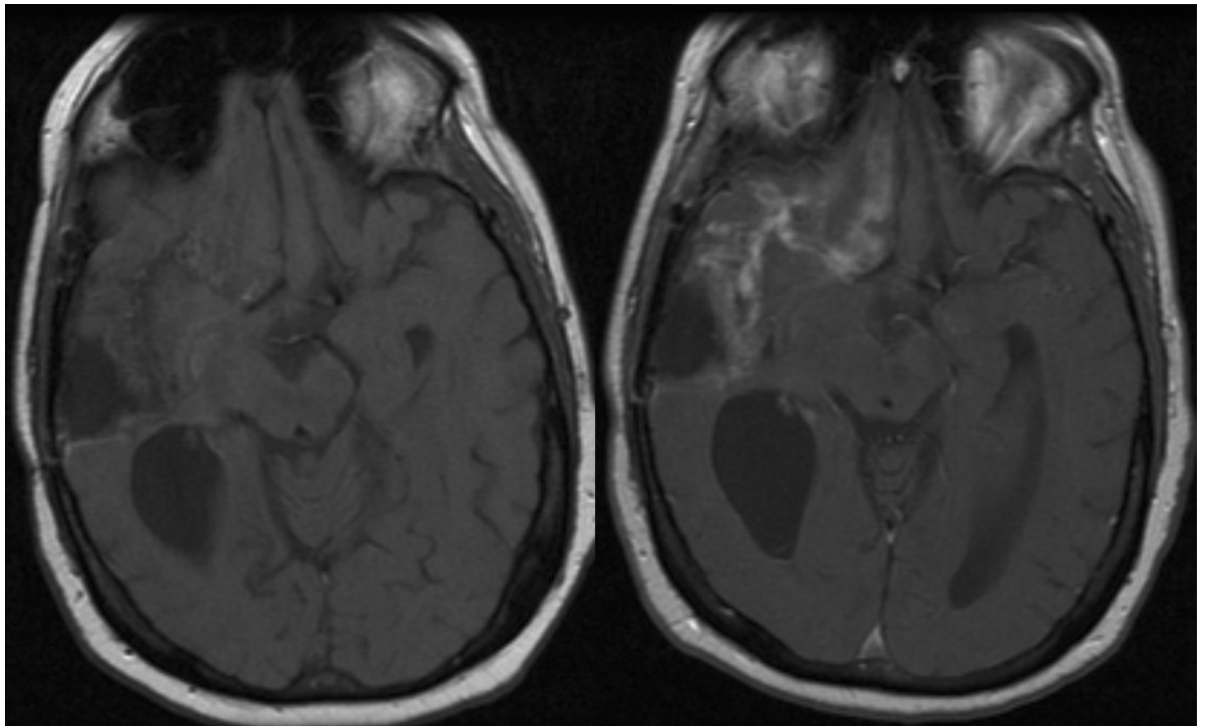


Figure 1.13. Magnetic resonance imaging showing features of an anaplastic astrocytomas (rad.usuhs.mil/rad/who/JPA.html) post-contrast image reveals a large enhancing irregular, mass in the left temporal lobe

Glioblastoma multiforme (GBM) tumours are defined as Grade IV and are amongst the most common glioma tumours. They can arise in one of two ways, either spontaneously without prior indication of a malignant lesion, or as a malignant progression from a lower grade lesion (Kleihues & Cavenee, 2000; Tso *et al*, 2006). GBMs are most prevalent in older adults (45-60 years), and usually arise in the frontal, temporal and insular regions of the cerebral hemispheres, although they occasionally may be located in the brainstem or cerebellum (Behin *et al*, 2003; Brat *et al*, 2008). GBMs are normally represented in MNR images as a grossly irregular hypodense heterogeneous mass, with various degrees of contrast-enhancement and vasogenic oedema (Figure 1.14). The most common presentation is as a ring-like enhancement surrounding a necrotic center (Behin *et al*, 2003, Brat *et al*, 2008).

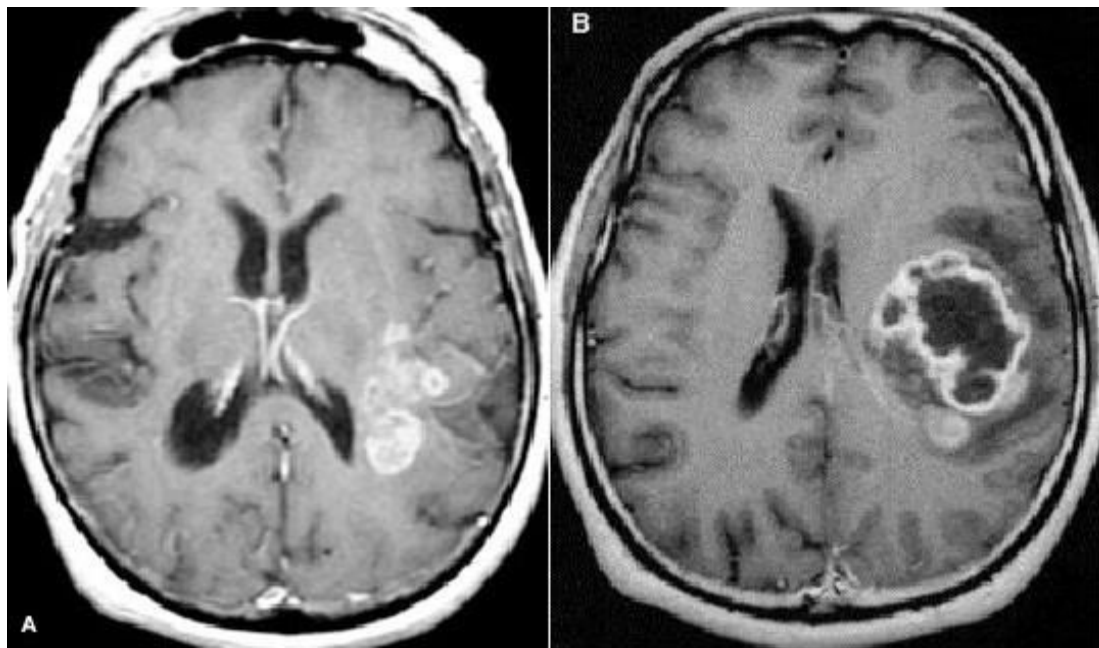


Figure 1.14. Magnetic resonance imaging features of glioblastoma multiforme (rad.usuhs.mil/rad/who/JPA.html) (A) post-contrast image revealing a multilobulated, enhancing mass in the right temporal lobe. (B) glioblastoma; tumour is an irregularly shaped, heterogeneous mass with central necrosis and ring-like contrast enhancement surrounded by oedema.

Cancerous tissues generally contain high levels of stress proteins, particularly members of the chaperone and heat shock protein families (Ciocca and Calderwood, 2005; Graner *et al*, 2007). The response of cells to such stress conditions is in part dependent on the concentration of heat shock proteins in the cell prior to stress. The intensity and duration of the stress will have an influence on the eventual outcome for the cell of either survival, apoptosis or necrosis (Mosser and Morimoto, 2004; Multhoff, 2007). During a stress response, while total protein synthesis is inhibited, the synthesis of specific heat shock proteins is elevated in recovering cells (cytoprotected state) (Lindquist and Craig, 1988). Initiation of apoptosis induces the expression of heat shock proteins, which continues until protein synthesis ceases (Mosser and Morimoto, 2004; Parsell *et al*, 1994).

Protein-damaging environmental and physiological stresses induce the expression of heat shock proteins that are often constitutively over-expressed in various cancer cells and are also involved in tumour cell proliferation, cellular death, metastasis and differentiation, suggesting that they may be a contributing factor in tumourigenesis (Calderwood *et al*, 2006, Ciocca and Calderwood, 2005; Graner *et al*, 2007; Mosser and Morimoto, 2004; Powers *et al*, 2007). It has been widely reported that elevated levels of *HSPA/HSP70*, *HSPB/HSP27* and *HSPC/HSP90*, either as combinations or individually, have been observed in breast, uterine, renal, brain, endometrial and osteosarcoma cancers (Graner *et al*, 2007; Jolly and Morimoto, 2000).

Numerous publications have studied heat shock protein expression and heat stress characterization in a wide range of tumour cell types (Calderwood *et al*, 2006; Ciocca and Calderwood, 2005; Li and Lee, 2006) However, studies are rare in different types of brain tumours (Fuse, 1991; Hermisson *et al*, 2000; Wang *et al*, 2006). A study

undertaken by Graner *et al*, (2007) examined changes in heat shock protein gene expression and localization of heat shock proteins in a number of brain tumour cell lines both at intracellular and cell surface/extracellular levels under normal conditions and the effect of heat shock on expression and localisation (Table 1.7).

Table 1.7. Summary of inducible HSPA/HSP70 expression in select brain tumour cell lines (relative to unstressed cells or to non-heat shock protein expression such as actin) (adapted from Graner *et al*, 2007).

| Cell Line | HSPA/HSP70 expression (Western blot) | | Cell surface HSPA/HSP70 expression (flow cytometry) | | Extracellular HSPA/HSP70 release (ELISA) | |
|---|---|-------------|---|-------------|--|-------------|
| | Constitutive | Heat Stress | Constitutive | Heat Stress | Constitutive | Heat Stress |
| D54MG Adult malignant | + | ++ | + | ++ | + | ++ |
| D392MG Adult malignant glioma | +/- | +++ | ++ | +++ | ++ | +++ |
| D341MED Pediatric medulloblastoma | ++ | +/- | + | ++ | + | +++ |
| SMA560 Murine anaplastic astrocytoma | +/- | ++++ | +/- | ++ | + | ++++ |

(+/-, Detectable expression; +, moderate expression; ++ ~~+~~ ++++, high to very high expression)

Although a number of heat shock proteins were examined, the most significant effects of heat shock related to HSPA/HSP70, and a clear increase in *HSPA/HSP70* expression following heat shock was reported by these authors. Western blot analysis showed no consistent correlation between the different cell lines regarding *HSPA/HSP70* expression as a result of heat shock. D392MG (adult malignant glioma) (Ostrowski *et al*, 1991) and SMA560 (a murine anaplastic astrocytoma) (Sampson *et al*, 1997; Serano *et al*, 1980) cell lines showed significantly higher levels of *HSPA/HSP70* gene expression compared to D54MG (derived from line A172, adult malignant glioma) (Giard, 1973) and D341MED (pediatric medulloblastoma) (Friedman *et al*, 1988) after heat shock, while the converse was observed in untreated cells. In most cell lines,

HSPA/HSP70 was present on the surfaces of the cells at normal temperature, and increased when the cells were exposed to heat shock and allowed to recover for a period of 24h. The fold increases ranged from ~1.5- to ~20-fold after heat shock. The results from the spent media (media that the cells were grown in) for HSPA/HSP70 is between 1.5 – 10 fold higher after heat shock, which could not be attributed to cell death as the number of viable cells was the same before and after heat shock (Graner *et al*, 2007). Previous work by Guzhova *et al*, (2001), examined the release of HSPA/HSP70 into culture media in human glioblastoma T98G cells and found a greater release from cells subjected to heat shock, compared to that from cells under normal conditions.

Stress induced by heat shock produced a substantial variation in heat shock responses in different brain tumour cells, some of which showed a significantly higher level of expression after heat shock while, conversely, expression in some brain tumour cells was high in the absence of heat shock (Calderwood *et al*, 2006).

The overexpression of heat shock proteins in numerous cancers, including glioma, is associated with tumour cell proliferation, metastasis, invasion, differentiation and death (Nylandsted *et al*, 2000). In some cancers, the overexpression of heat shock protein genes is correlated with poor prognosis and a reduced positive response to therapy (Calderwood *et al*, 2006). HSPA/HSP70 has been associated with higher cell proliferation, with the worst prognosis in patients suffering from breast cancer (Ciocca *et al*, 2003). Overexpression of heat shock proteins requires the activation of heat shock transcription factor 1 (HSF1), which is also upregulated in cancer cells and is involved in invasion and metastasis (Ciocca and Calderwood, 2005).

1.3.2 Hypoxia

Hypoxia is a condition resulting from decreased cellular oxygen levels in tissues, and is associated with many diseases, such as pulmonary disease, heart disease, vascular disease and cancer. Research has shown that regions of acute/chronic hypoxia exist within the majority of solid tumours (Williams *et al*, 2001). In cellular hypoxia, tumours require additional blood vessels, a condition which is evident in many neoplasms. Hypoxia induces transcriptional activation of many genes that affect cellular metabolism and also promotes neoangiogenesis (the formation of new or recent blood vessels) (Duffy *et al*, 2003). Cells are able to sense and respond to decreased oxygen levels through the conserved hypoxic response pathway involved in tumourigenesis. Exposure to decreased oxygen levels initiates the hypoxic response pathway by the regulated expression of hypoxia inducible transcription factor-1 (HIF-1). HIF-1 is a transcription factor which, under hypoxic conditions, specifically binds to the region 5' – RCGTG- 3' of the hypoxia-responsive element (HRE) in the promoter of various hypoxia-inducible genes such as heat shock protein gene families, erythropoietin (EPO); vascular endothelial growth factor (VEGF) (Simiontonaki *et al*, 2008) and von hippel-lindau (VHL) (Baird *et al*, 2006; Gombos *et al*, 2011; Huang and Bunn, 2003). HIF-1 is a member of the basic-helix-loop-helix (bHLH)-PAS family. PAS is an acronym for the first three members recognised, being period homolog (Per), aryl hydrocarbon receptor nuclear translocator (ARNT) and single minded homolog (Sim) (Huang and Bunn, 2003). HIF-1 is a heterodimer composed of one of three subunits, HIF-1 α ~120 KDa, HIF-2 α ~118 KDa and ~72 KDa HIF-3 α subunits and a 91 – 94 KDa HIF-1 β /ARNT subunit (Baird *et al*, 2006; Gombos *et al*, 2011; Huang and Bunn, 2003). Under normal oxygen conditions (normoxia), both HIF-1 α and HIF-1 β mRNAs and proteins are constitutively expressed (Baird *et al*, 2006; Wang *et al*, 1995).

Many of the hypoxia-inducible genes are up-regulated during hypoxia, thus increasing oxygen transport to hypoxic tissues by promoting angiogenesis and also promoting cell proliferation and survival. However, failure to adapt to decreased oxygen levels will ultimately result in cell death via apoptosis (Bruick, 2003). Angiogenesis is associated with metastasis, and as tumours grow, some cells become detached from the tissues nutrient supply limiting the delivery of oxygen and nutrients to those cells (Duffy *et al*, 2003). This results in cellular hypoxia and metabolic stress inducing changes in transcriptional regulation, promoting growth of highly permeable blood vessels and facilitating the passage of tumour cells into the circulatory system. The response of cancer to hypoxia not only maintains the survival and growth of tumours, but promotes tissue invasion and metastasis through angiogenesis (Duffy *et al*, 2003).

Activation of heat shock proteins plays a crucial role in adaptation by cells to hypoxic conditions and in tolerating the oxidative stress of reoxygenation (Baird *et al*, 2006; Semenza 1999). Heat shock proteins are regulated by heat shock factors and are linked to the oxygen-sensing and heat shock pathways. The activity of the heat shock pathway is controlled by the trimerization and post translational modification of heat shock factor protein subunits (Baird *et al*, 2006; Orosz *et al*, 1996). Transcription of heat shock factors is controlled by HIF-1 which is increased during hypoxia, increasing the cellular abundance of heat shock factors and the sensitivity of the heat shock pathway, thus maximising the production of protective heat shock proteins (Baird *et al*, 2006). During hypoxia, heat shock factors are up-regulated due to the direct binding by HIF-1 to HIF-1 response elements. The up-regulation of heat shock factors are essential for the increase of heat shock protein transcripts during hypoxia and reoxygenation (Baird *et al*, 2006).

1.3.3 Post-mortem conditions

In cases of unexplained death, one of the most important investigative factors is the accurate and precise determination of the time interval since death occurred, which is usually referred to as the post-mortem interval (PMI). There has been much debate as to the precise moment when death actually occurs. Various methods involving many different scientific disciplines, such as forensic medicine, anthropology, physiology, molecular biology and entomology have been employed in determining an accurate post-mortem interval, and the application of these methods largely depend on whether the PMI is assumed to be short (hours/days), medium (weeks) or long term (years) (Mathur and Agrawal, 2011). To date, the precise time of death cannot be determined with complete accuracy or certainty. Broadly speaking, there are two phases of death: somatic death, which is the cessation of the integrated functioning of an individual at a physiological level, and molecular or cellular death, which is the cessation of respiration and the normal metabolic activity in the tissues and cells. However, there has been much debate surrounding cellular death as to when this actually occurs. Various methods are currently employed in determining PMI. These include temperature measurements made post-mortem (Henssge and Madea, 2004), the use of biochemical markers such as protein fractions and enzymes (Fountoulakis *et al*, 2001; Sabucedo and Furton, 2003), potassium concentration in the vitreous humor of the eye (Madea and Rodig, 2006) eye temperature decrease (Kaliszan *et al*, 2010) and post-mortem muscle proteolysis (the relaxation of muscles following rigor mortis) (Madea and Henssge, 1990).

The most commonly used method for the determination of short term PMI by pathologists is based on the cooling of the body and involves the measurement of the

body core temperature at various time points. This requires the direct measurement of the intra-abdominal temperature and applying this to Newton's law of cooling (Besson, 2010; Newton, 1701) which states –the rate of cooling of a body is determined by the difference between the temperature of the body and that of its environment‖ (Henssge, 1988; Henssge and Madea, 2004; Marshall and Hoare, 1962). Various algorithm / nomogram models have been suggested for its use, and the current preferred model is the –temperature time of death relating nomogram‖ (Figure 1.15) developed on the basis of physical considerations, and the two-exponential term of Marshall and Hoare (1962).

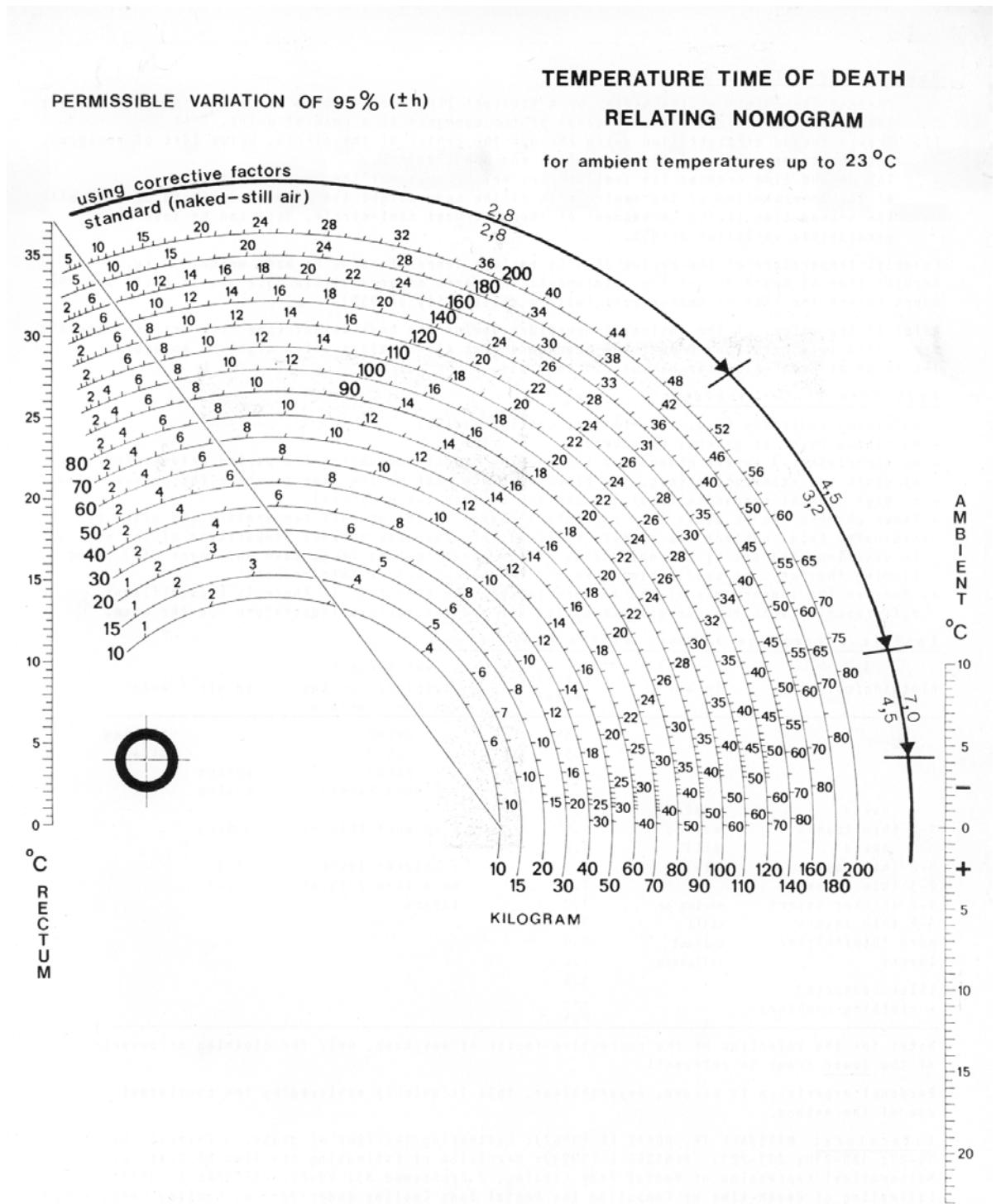


Figure 1.15. The temperature-time of death relating nomogram (adapted from Hessenge and Madea, 2004).

Death-time (t) is expressed by:

$$\frac{T_{\text{rectum}} - T_{\text{ambient}}}{37.2 - T_{\text{ambient}}} = 1.25 \exp(Bt) - .25 \exp(5Bt); B = -1.2815 (\text{kg}^{-.625}) + .0284$$

The nomogram is related to the chosen standard of a naked body lying in still air. Cooling conditions that differ from the chosen standard may be proportionally adjusted by corrective factors as shown in Table 1.8 of the real body weight (Hessenge and Madea, 2004).

Table 1.8. Empiric corrective factors of the body weight (adapted from Hessenge and Madea, 2004).

| Dry clothing / covering | In air | Corrective Factor | Wet clothing / covering wet body surfaces | In air / water |
|--------------------------------|-------------------|--------------------------|--|-----------------------|
| | | 3.5 | Naked | Flowing |
| | | .5 | Naked | Still |
| | | .7 | Naked | Moving |
| | | .7 | 1-2 thin layers | Moving |
| Naked | Moving | .75 | | |
| 1-2 thin layers | Moving | .9 | 2 or more thicker | Moving |
| Naked | Still | 1.0 | | |
| 1-2 thin layers | Still | 1.1 | 2 thicker layers | Still |
| 2-3 thin layers | | 1.2 | More than 2 thicker layers | still |
| 1-2 thicker layers | Moving or still | 1.2 | | |
| 3-4 thin layers | | 1.3 | | |
| More thin/thicker layers | Without influence | 1.45 | | |
| Thick bedspread & clothing | | 1.8 | | |
| combined | | 2.4 | | |

Although this is the preferred method, there are still factors that cause much controversy and doubts regarding the complete accuracy of this method. This method assumes a constant ambient temperature, but it has been suggested by Althaus and Henssge (1999) that, if there is a rapid increase or decrease in ambient temperature, the PMI based on this method would not be totally reliable.

One area that has not been fully investigated with regards to PMI is the persistence of gene transcripts in post mortem tissue. In the period immediately following death there are a number of cellular functions that persist, such as continued cell division, continued reactivity of muscle fibres to stimuli, mechanical or electrical stimulation (Jones *et al*, 1995; Madea 1992; Schleyer, 1963) and continuation of enzymatic activities such as adenylyl cyclase which has conserved basal activity in neural tissue up to 48h after death (Palego *et al*, 1999). During the early post mortem period, the amount of RNA in cells rapidly changes depending on pathophysiological changes of the microenvironments involved in the death process (Maeda *et al*, 2010). However, some genetic reactions continue during the early post-mortem period, such as supravital reactions, the reactions that occur within the body from somatic to cell death, which result in either an increase or decrease in mRNA transcripts, which may modify the biological status at time of death (; Ikematsu *et al*, 2008; Maeda *et al*, 2010).

In general, the cause of death is determined by evaluating functional and morphological changes of the viscera (internal organs), tissues and cells following a fatal insult (Maeda *et al*, 2010). Death due to a functional mechanism such as fatal disease, injury or chemical insult, may produce few morphological changes, however the status of cell functions may be preserved in the mRNA pattern indicated by precise up / down regulations of gene expression which occurs rapidly after death (Maeda *et al*, 2010;

Zhao *et al*, 2009). Studies undertaken by Ikematsu *et al* (2008) have demonstrated a significant tissue- specific increase or decrease in mRNA levels in the early post mortem period of < 12h. The study was undertaken using mechanically asphyxiated mouse lung and brain after somatic death, for the detection of early response genes including myelocytomatosis oncogene, C-MYC, inducible-nitric oxide synthase 2, i-NOS, and FBJ osteosarcoma oncogene, C-FOS (Ikematsu *et al*, 2008). Rapid changes in mRNA levels, depending on the pathophysiology of death could be useful to investigate acute violent deaths such as electrocution, asphyxiation or in cases of sudden death.

The measurement of gene expression at the mRNA level in post-mortem tissue can potentially provide useful information relevant to the determination of a more accurate post-mortem interval and the possible circumstances, mechanisms and diagnosis surrounding or leading to the cause of death.

Studies by Anderson *et al*, (2005); Bauer *et al*, (2003) and Maarti, (2004) have shown that RNA remains largely intact for a considerable time period (up to 24 to 48 h) after death under appropriate ambient conditions. Furthermore, assessing mRNA levels by RT-PCR is believed to produce more accurate results than other techniques, such as comparison with levels of ribosomal 18S and 28S rRNAs. Therefore, measurement of cellular mRNA levels could provide information about RNA quality in tissues of relevance to PMI determination as well for research or diagnostic purposes, such as wound age determination.

A number of recent studies (Anderson *et al*, 2005; Bauer *et al*, 2003; Maarti, 2004) have shown that there is the potential to determine post-mortem interval in a forensic context by measuring the levels of total RNA populations and the transcripts of specific genes

in the early post-mortem period. These studies were specifically undertaken in relation to the identification of body fluids, the age of blood and semen stains, the age of wounds (Anderson *et al*, 2005; Bauer *et al*, 2003) and the functional status of cells and organs for the purpose of forensic pathology (Maarti, 2004). Studies have also demonstrated, across a wide range of both human and rat tissue types, that the molecular integrity of RNA (the 28S and 18S ribosomes) and targeted gene transcripts can remain relatively high in post-mortem tissue for up to periods of 148 h (brain tissue; Inoue *et al*, 2002); 48 h (bone, Kuliwaba *et al*, 2005); 72 h (lungs, heart and muscle tissue, De Paepe *et al*, 2002; Heinrich *et al*, 2007; Inoue *et al*, 2002); and 24 h (liver, kidney and spleen tissue, Heinrich *et al*, 2007; Inoue *et al*, 2002). The results were all obtained under ambient temperatures or at 4°C using real time quantitative reverse transcription polymerase chain reaction (RT-PCR).

Gopee and Howard (2007) demonstrated that, over a time course of post-mortem intervals up to 60 min, skin excised from 10 month old hairless mice showed minimal variation in terms of the integrity of total RNA and also the mRNA of the genes C-MYC, CYR61, HIF-1 α , C-CND1 and one housekeeping gene 18S ribosomal RNA.

Overall these studies indicate that the cellular integrity of mRNA is unlikely to change significantly in the early post-mortem period, thereby allowing the opportunity to elicit *de novo* gene expressions of specific target genes in response to mild heat shock.

A study undertaken by Pardue *et al*, (2007) compared the induction and the levels of HSPA/HSP70 and heat shock cognate HSPA8/HSC70 proteins and mRNAs in human brain (in cases of sudden or unexpected death with no known agonal stresses pre-mortem) with those in brains of non-stressed and heat shocked rats. Post-mortem

intervals ranged from 3.5 – 22 h in human brain samples, 3 h for non-stressed rat brain at room temperature and 0 – 24 h for rat brains that were subjected to heat shock at 3, 5, 8, 12 and 24 h. Results indicated that the levels of HSPA/HSP70 proteins were 43-fold higher in human brain than those in non-stressed rat brain and 14-fold higher than those subjected to heat shock. HSPA8/HSC70 results were less dramatic, showing only a 1.5-fold increase in that of human brain samples compared to that of non-stressed rat brain. Consistent with the presence of the HSPA/HSP70 protein, *HSPA/HSP70* mRNA levels were also found to be 8.5-fold higher in human brain than in non-stressed rat brain. Clearly, the overall conclusion is that HSPA/HSP70 protein levels are significantly higher in humans who die suddenly and are not subjected to agonal stresses pre-mortem than in rats either non-stressed pre-mortem or subjected to heat shock post-mortem.

HSPA/HSP70 has been linked with post-mortem events due to its association with BAG-1 (Bcl-2-associated athanogene-1) and CHIP (carboxyl terminus of the Hsc70-interacting protein). BAG-1 contains a domain within HSPA/HSP70 nucleotide-exchange activity that is presumed to assist molecular chaperones in the removal of aberrant proteins from the cytosol (Curcio *et al*, 2006; Lanneau *et al*, 2007; McClellan *et al*, 2005). BAG-1 has a capacity to inhibit apoptosis and modulates the chaperone activity of HSPA/HSP70 and heat shock cognate 70 (HSPA8/HSC70) by binding to their ATPase domains (Luders *et al*, 2000b; Seidberg *et al*, 2003; Stuart *et al*, 1998; Takayama *et al*, 1997;). BAG-1 appears to alleviate cellular stress by joining HSPA/HSP70 and other chaperones to the ubiquitin proteasome system facilitating degradation of oligomeric species. However, during the agonal state linking of BAG1 and HSPA/HSP70 to the ubiquitin proteasome system can be affected by various pre and post-mortem variables (Curcio *et al*, 2006).

1.4 Aims of the Research

The expression of *HSPA/HSP70* is up-regulated by various cellular stress factors, including cancer, hypoxia and post-mortem conditions. The main research aims of this project were to explore and measure the effects of these factors further, using human and rat tissues and cell lines. Experiments were devised to characterise *HSPA/HSP70* expression in normal and glioma cell lines, and under hypoxic conditions. Expression was also characterised in post mortem tissues at different stages of the early post mortem interval, to determine whether *HSPA/HSP70* was capable of *-de novoll* expression in response to cellular signals for its use as an early post-mortem marker to estimate PMI. In terms of the potential for clinical application, this research also attempted to assess the possible use of *HSPA/HSP70* as a prognostic marker.

To provide structure to the presentation of results in this thesis, these objectives can be individually summarised as follows:

1. To characterize the level of *HSPA* expression in normal human cell lines and normal human brain tissue, compared to that of glioma cell lines and tumourous brain tissue;
2. To characterize the level of *HSPA* transcription in response to hypoxia in normal and glioma cell lines;
3. To characterize the level of *HSPA* transcription in post-mortem brain tissue ;
4. To characterize the level of *HSPA* transcription in response to heat shock applied to the brain tissue in the early post-mortem period;

5. To characterize the level of *HSPA* transcription in response to multiple heat shocks applied to the brain tissue in the early post-mortem period; and
6. To determine the potential for *HSPA* to be used as an early post-mortem marker.

Chapter 2

Materials and Methods

2.1 Cell Culture

2.1.1 Tissue samples, cell lines and culture conditions

Tumour and normal (control) brain tissue samples were obtained from patients admitted to the Royal Preston Hospital, UK. Ethical approval from both the North Manchester Research Ethics Committee Ref: 06/Q1406/104 and the Ethics Committee at the University of Central Lancashire were obtained prior to work being carried out on tissue samples. Tumour tissue samples were obtained from glioma cancer patients, and normal (control) tissue samples were obtained from patients who required resection of normal brain for purposes other than primary glioma treatment. Written consent was obtained prior to tissue samples being used in this investigation. For each patient, brain tissues samples were surgically dissected and immediately frozen at -80°C and stored for analysis (performed by neurosurgeons and the pathology department at the Royal Preston Hospital, UK). In total, 18 tissue samples were used in this study.

Human brain cell lines grade I astrocytoma, 1321N1 from European Collection of Cell Cultures, ECACC, (UK), grade II/III oligodendroglioma GOS-3 from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DMSZ, (Germany), grade IV glioblastoma, U87-MG from ECACC (UK) and normal human astrocytes, NHA from Lonza (UK) were used in this study. 1321N1 and GOS-3 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% foetal bovine serum (FBS) and with 2 mM and 4 mM L-glutamine respectively, while U87-MG cells were cultured in Eagle's minimum essential medium (EMEM; Sigma) supplemented with 2 mM L-glutamine, 10% FBS, 1 mM sodium pyruvate and 1% (v/v) nonessential amino acids, NEAA, (Sigma). NHA cells were cultured in astrocyte

medium (AM) supplemented with 15 ml (3%) of FBS, 0.5 ml (0.1%) ascorbic acid, 0.5 ml recombinant human epidermal growth factor, rhEGF, 0.5 ml gentamicin amphotericin-B, GA-1000, 1.25 ml insulin and 5 ml L-glutamide.

All cells were of human origin without infectious viruses or toxic products. Routine mycoplasma testing was performed at the University of Central Lancashire on all cell lines and strains used in the study. Cells were received as frozen ampoules in 1 ml plastic cryotubes containing cells suspended in appropriate freezing medium and 10 % (v/v) dimethyl sulphoxide (DMSO), as supplied by commercial laboratories.

2.1.2 Media and Reagents

Complete medium for cell growth was aseptically prepared for each cell line according to the supplier's recommendations with the addition of specific supplements (Table 2.1).

Table 2.1 Media and supplements for each cell line used in this thesis.

| Cell Line | Medium | Supplements |
|-----------|---|--|
| 1312N1 | Dulbecco's Modified Eagle's Medium (DMEM) | 10% FBS 2 mM L-glutamine |
| GOS-3 | Dulbecco's Modified Eagle's Medium (DMEM) | 10% FBS 4 mM L-glutamine |
| U87-MG | Eagle's Minimum Essential Medium (EMEM) | 10% FBS 2 mM L-glutamine 2 mM non-essential amino acids 1 mM sodium pyruvate |
| NHA | Astrocyte Basal Medium (ABM) | 0.5 ml recombinant human epidermal growth factor (rhEGF) 1.25 ml insulin 0.5 ml ascorbic acid 0.5 ml gentamicin amphotericin-B (GA-1000) 5.0 ml L-glutamine 3% FBS |

2.1.3 Preparation of Media

The media for cell lines were prepared according to the individual cell line requirements, based on the ECACC / DMSZ / Lonza recommendations and all additional supplements were added in accordance with the supplier's recommendations. Supplemented media were labeled with date of preparation and stored for up to a maximum of two weeks at 4°C. All reagents and chemicals used for cell culture are

listed in Table 2.2. Prior to inoculation of the complete media with the cell lines, the complete medium for each cell line was pre-incubated overnight to ensure that no contaminants, mycoplasma or toxic products were present.

Table 2.2 Reagents and chemicals used in cell culture.

| Reagents | Supplier | Components |
|------------------------------|-----------|---|
| Foetal bovine serum | Gibco BRL | Heat inactivated foetal bovine serum |
| Non-essential amino acid | Sigma | 100x non-essential amino acid |
| Trypsin EDTA | Sigma | 0.5 g porcine trypsin 0.2 g EDTA |
| L-glutamine | Sigma | 200 mM L-glutamine |
| Phosphate buffer saline 0.1M | Sigma | 8 g/l sodium chloride 0.2 g/l potassium chloride |
| DMSO | Sigma | Dimethyl sulfoxide 99.5% |
| Trypan blue (0.4% v/v) | Sigma | 0.81% w/v sodium chloride 0.06% w/v potassium phosphate dibasic |

2.1.4 Resuscitation of Cells

Complete media for cell lines were pre-warmed to 37°C before the frozen ampoules containing the cells were added and thawed rapidly in a 37°C water bath. Thawed cells were immediately rinsed by resuspension in 4 ml of appropriate growth medium to remove the freezing medium and centrifuged at 150 g for 5 min. The supernatant was discarded and the cells were resuspended in 3 ml of the appropriate medium and then aliquoted into 2 x 25 cm² flasks together with 5 ml of the appropriate medium and mixed manually. Flasks were suitably labeled with the cell line, passage number and then incubated at 37°C with 5% CO₂ in 95% filtered air.

2.1.5 Subculture

Following overnight incubation, the cells were observed using a phase contrast microscope for a monolayer growth of approximately 70–80% confluency and to confirm the absence of bacterial and fungal contaminants. Depending on observations, cells were either trypsinised or subjected to hypoxia treatment. However, for slow growing cell lines, the medium was changed every 48 h after incubation to ensure sufficient nutrients for cell growth.

2.1.6 Hypoxia Treatment

Cells were cultured in 75 cm² tissue culture-treated polystyrene flasks (Sigma). Hypoxic conditions were induced by exposing confluent cells to nitrogen (100 %) for 30 min (Kay *et al*, 2007). After treatment, the cells were collected at various recovery periods (0, 3, 6 and 24 h) for experiments concerning gene expression, immunofluorescence and flow cytometry assays.

2.1.7 Trypsinisation

The culture medium was removed and the cells were washed once with 5 ml of 1 x phosphate buffered saline (PBS) (0.1 M, pH 7.4) to remove any excess culture medium. Trypsin EDTA 1 x (1ml/ 25cm² flask, 2ml/ 75cm² flask) was added to each flask and cells were incubated under normal conditions for 5-10 min at 37°C (atmosphere of 95% O₂, 5% CO₂). The cells were examined using a phase contrast microscope to ensure that all the cells had detached and were floating. Cells were resuspended in 2ml/ 25cm² flasks or 4ml/ 75cm² flasks of the appropriate medium to inactivate the trypsin. The cell suspension was transferred to a 15 ml centrifuge and centrifuged (ALC PK120) at

1000 rpm for 5 min. The supernatant was discarded and the pellet resuspended in 1 ml of medium followed by gentle pipetting to aid resuspension.

2.1.8 Cell Quantification

Cell quantification involved first adding 20 μ l of the cell suspension and 80 μ l of trypan blue (Freshney, 1987) (1:5 dilutions) to a 1.5 ml microfuge tube and the suspension mixed by gently pipetting. Cells were observed and cell concentration calculated by the use of a Neubauer haemocytometer. Samples were prepared by attaching a cover slip using slight pressure until Newton's refraction rings appeared (rainbow-like rings under the cover-slip). Both sides of the chamber were filled (approx. 10 μ l) with cell suspension / trypan blue solution, ensuring no air bubbles were present, and samples viewed under a light microscope using x 20 magnification. The number of viable cells (seen as bright cells) were counted in the central square (gridded 5 x 5 squares) and the 4 squares above and below, left and right of the central square, followed by calculation of the average number of cells per large square. This number = $\times 10^4$ and equalled the number of cells per ml within the cell suspension / trypan blue solution. During this calculation, it was noted that, due to the addition of trypan blue, the number of cells had to be $\times 5$ to correct for the dilution. The cell suspension was then diluted to a concentration of 1×10^5 cells/ml. The dilution factor was calculated using the following equation:

$$\text{Dilution factor} = \frac{\text{Concentration of original cell suspension}}{\text{Concentration of required cell suspension}} \times \text{Volume}$$

2.2 mRNA Isolation

mRNA was isolated using mRNA Isolation Kit (Roche-applied-science, Germany Cat. No. 11 741 985 001). Reagents were mixed according to the manufacturer's instructions using a vortex mixer and briefly centrifuged prior to commencing this procedure. This kit works on the basis that the poly (A) tail of mRNA hybridises to a biotin-labelled oligo(dT) probe. Streptavidin-coated magnetic particles capture the biotinylated hybrids and, with the aid of a magnetic separator, the magnetic particles are captured. The fluid is then removed by washing the particles with washing buffer and finally the mRNA is eluted from the particles by incubation with redistilled water.

Table 2.3. Reagents, composition and quantity provided mRNA isolation Kit (Roche).

| Reagent | Composition | Quantity |
|--|--|------------|
| Lysis Buffer | 0.1 M tris buffer, 0.3 M lithium chloride (LiCl), 10 mM ethylenediaminetetraacetic acid (EDTA), 1% lithium dodecylsulphate, 5 mM dithiothreitol (DTT) pH 7.5 | 1 x 100 ml |
| Streptavidin-coated magnetic particles (SMP's) | 10mg/ml in 50 mM hepes, 0.1% bovine serum albumin, 0.1% chloracetamide, 0.01% methylisothiazolone, pH 7.4 | 1 x 1.7 ml |
| Oligo(dT) ₂₀ probe biotin labelled | 100 pmol biotin labelled oligo(dT) ₂₀ per µl of redistilled water | 1 x 66 µl |
| Washing buffer | 10 mM tris buffer, 0.2 M LiCl, 1 mM EDTA, pH 7.5 | 1 x 50 ml |
| Double redistilled water, RNase free | RNase-free H ₂ O | 1 x 4 ml |
| Storage buffer | 10 mM tris buffer, 0.1% chloracetamide, 0.01% methylisothiazolone, pH 7.5 | 1 x 7 ml |

mRNA was isolated from 2×10^6 cells following the manufacturer's protocol as shown in the schematic diagram (Figure 2.1). The volumes and composition of each reagent and buffers provided within the kit are detailed in Table 2.3, whilst the specific measures used during each extraction procedure are shown in Table 2.4.

Cells were washed by suspension in $3 \times 500 \mu\text{l}$ of ice cold Phosphate Buffer Saline (PBS, 0.1 M) to remove excess media which could potentially interfere with UV spectrophotometric measurements. The suspension was centrifuged at 10,000 g for 3 min at room temperature and the supernatant discarded. To the cell pellet, 500 μl of lysing buffer (0.1 M Tris buffer, 0.3 M LiCl, 10 mM EDTA, 1% (w/v) lithium dodecylsulphate, 5 mM dithiothreitol, DTT, pH 7.5) was added, followed by mechanical shearing of cells, achieved by passing samples through a 21 gauge needle (six times). An aliquot of 0.5 μl biotin labelled oligo(dT)₂₀ probe (100 pmol biotin-labelled oligo(dT)₂₀ per μl of molecular biology grade H₂O) was added to the cell lysate and allowed to hybridise with mRNA for 10 min at room temperature. Simultaneously, 50 μl of streptavidin magnetic particles (10 mg/ml of suspension in 50 mM Hepes, 0.1 % (w/v) bovine serum albumin, 0.1 % (w/v) chlorace tamide, 0.01 % (w/v) methylisothiazolone, pH 7.4) was aliquoted into a 1.5 ml sterile microfuge tube and separated from the storage buffer by placing in a magnetic separator, after which the storage buffer was discarded. The particles were cleansed by resuspension in 70 μl of lysing buffer (0.1 M Tris buffer, 0.3 M LiCl, 10 mM EDTA, 1% (w/v) lithium dodecylsulphate, 5 mM DTT, pH 7.5). Following magnetic separation and disposal of the supernatant, the prepared particles were resuspended in the dT-mRNA hybrid mixture; after brief vortexing, the resultant suspension was incubated for 5 min at 37°C to achieve immobilisation. Following incubation, the hybrid-linked particles were magnetically separated from this fluid and the supernatant was discarded. The remaining particles were then washed by resuspension in $3 \times 200 \mu\text{l}$ of washing buffer

(10 mM Tris buffer, 0.2 M LiCl and 1 mM EDTA, pH 7.5). Upon disposal of the final supernatant, mRNA was eluted from the particles by resuspension in 10 μ l of redistilled water, mixing and incubation for 2 min at 65°C. After magnetically separating the particles from the fluid, the resulting supernatant (mRNA) was stored at -20°C in an RNase free microfuge tube ready for quantification and analysis.

Table 2.4. Volumes of reagents used for mRNA isolation as per manufacturers' protocol.

| | Amount of Tissue (mg) | | Number of Cells | | | | |
|--|-----------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| | 200 | 50-100 | 1×10^8 | 2×10^7 | 1×10^7 | 2×10^6 | 2×10^5 |
| Volume of lysis buffer | 3 ml | 1.5 ml | 15 ml | 3 ml | 1.5 ml | 0.5 ml | 0.1 ml |
| Volume of streptavidin magnetic particles | 300 μ l | 150 μ l | 1.5 ml | 300 μ l | 150 μ l | 50 μ l | 50 μ l |
| Volume of lysis buffer: streptavidin magnetic particle preparation | 500 μ l | 250 μ l | 2.5 ml | 500 μ l | 250 μ l | 70 μ l | 70 μ l |
| Volume of oligo(dT) ₂₀ probe biotin labelled | 3 μ l | 1.5 μ l | 15 μ l | 3 μ l | 1.5 μ l | 0.5 μ l | 0.5 μ l |
| Volume of washing buffer | 3 x 500 μ l | 3 x 250 μ l | 3 x 2.5 ml | 3 x 500 μ l | 3 x 250 μ l | 3 x 200 μ l | 3 x 200 μ l |
| Volume of redistilled water | 50 μ l | 25 μ l | 250 μ l | 50 μ l | 25 μ l | 10 μ l | 5 μ l |

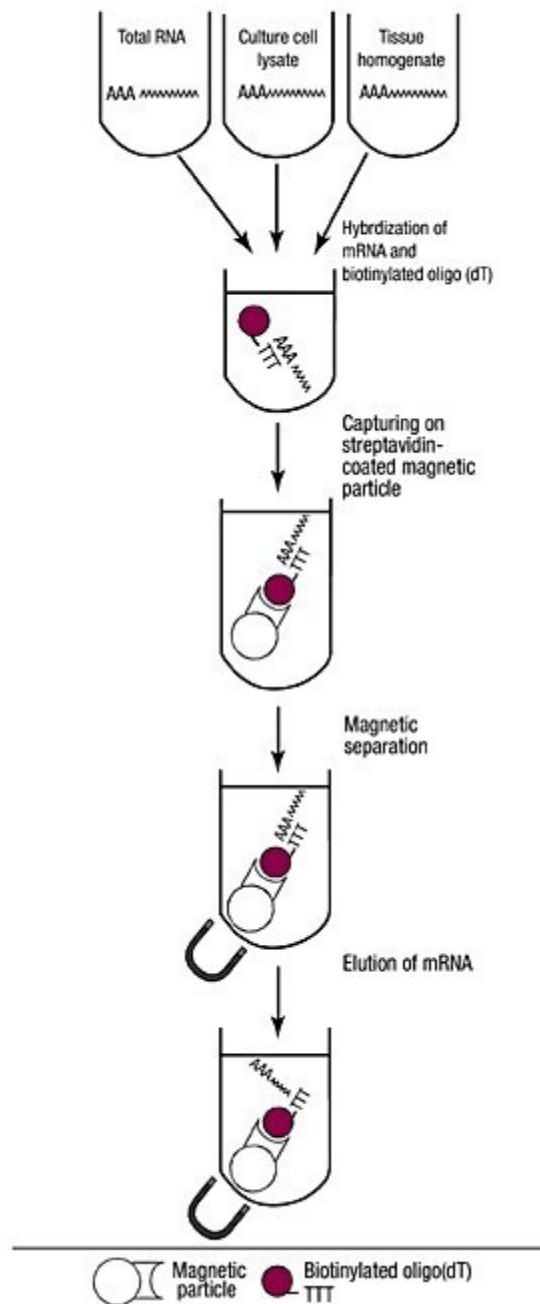


Figure 2.1 A diagrammatic representation of the principals involved in extracting mRNA from culture cells and tissue using the mRNA Isolation Kit (adapted from Roche mRNA Isolation Kit instruction manual).

2.2.1 Quantification of nucleic acids by UV spectrophotometry

Isolated mRNA was quantified by measurement of absorbance using the thermo spectrophotometer Helios gamma (Thermospectronics, UK) at wavelengths of 260 nm and 280 nm. Samples of mRNA (2 µl) were combined with 500 µl of TAE (Tris-acetate-EDTA) buffer (400 mM Tris, 0.01 M EDTA; pH 8.0). Spectrophotometric measurements were obtained and mRNA concentrations were calculated. The standard formula was based on an absorbance of one optical density (OD) unit at 260 nm = 50 µg/ml for dsDNA and 40 µg/ml for ssRNA. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of isolated nucleic acids. A ratio of 1.8 - 2.0 indicated the presence of pure single-stranded (ss) RNA. The concentrations of the isolated mRNA were calculated as follows

$$\text{Concentration mRNA (}\mu\text{g/ml)} = A_{260} \text{ reading} \times \text{dilution factor (250)} \times 40 \text{ (ssRNA)}$$

2.2.2 Analysis of Nucleic acid by agarose gel electrophoresis

Isolated mRNA was visualized on 2% agarose gels to determine whether RNA was intact or degraded. The 2% gel was prepared using 0.6 g of agarose powder (Geneflow, UK) dissolved in 30 ml TAE buffer (400mM Tris, 0.01 M EDTA; pH 8.3) and heated in a domestic microwave until a clear transparent solution had formed. The solution was poured into a prepared electrophoresis gel tank, combs inserted and left to solidify. TAE buffer was used to completely submerge the gel before the samples and a 100 bp ladder, as a molecular weight marker, were loaded into appropriate wells. Each sample loaded comprised 10 µl of extracted mRNA mixed with 2 µl of loading dye (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol and 40% w/v sucrose), together with 2 µl of 100 bp ladder mixed with 5 µl of loading dye. The gel was electrophoresed at 100 V for 25 min, followed by staining in 0.4 µg/ml ethidium bromide for 45 min and then

destained in distilled H₂O for approximately 10 min. The banding patterns were then visualized using a GENE GENIUS Bioimaging system and Gensnap software (Syngene, UK).

2.3 Complimentary DNA Synthesis (cDNA)

mRNA was reverse transcribed using First Strand cDNA Synthesis Kit, which harnesses enzymes isolated from Avian Myeloblastosis Virus (Roche-applied-science, Germany Cat. 11 483 188 001). The composition and volumes of all reagents provided are shown in Table 2.5, whilst the specific quantities required by each cDNA synthesis reaction are shown in Table 2.6. All reagents were kept on ice, mixed using a vortexer and briefly centrifuged prior to commencing this procedure.

Table 2.5 Reagents, composition and quantity of each reagent provided within the First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche).

| Reagent | Composition | Quantity |
|--|---|---------------------------------|
| 10 X Reaction Buffer | 100 mM tris buffer, 500 mM KCl; pH 8.3 | 1.05 ml |
| MgCl ₂ | 25 mM MgCl ₂ | 3 ml (3 vials; 1 ml in each) |
| Deoxynucleotide Mix | dATP, dCTP, dTTP, dGTP; 10 mM each | 210 µl |
| Gelatine | 0.5 mg/ml (0.05% [w/v]) gelatine | 210 µl |
| Oligo-p(dT) ₁₅ primer | 0.02 A ₂₆₀ U/µl (0.8 µg/µl) oligo-p(dT) ₁₅ primer sequence | 60 µl |
| Random primer p(dN) ₆ | 0.04 A ₂₆₀ U/µl (2 µg/µl) primer sequence | 60 µl |
| RNase inhibitor | 50 U/µl RNase inhibitor | 30 µl |
| AMV Reverse Transcriptase | AMV reverse transcriptase | 24 µl |
| Control Neo pa RNA | 0.2 µg/µl RNA sample; 1.0 kb in length with an additional 19-base 3'-poly(A) tail | 25 µl |
| Molecular biology-grade H ₂ O | RNase-free H ₂ O | 2 ml (2 vials; 1 ml in each) |

A master mix of 2 μ l of 10 X reaction buffer (500 mM KCl; pH 8.3), 4 μ l of $MgCl_2$ (25 mM), 2 μ l of deoxynucleotide mix (dATP, dCTP, dGTP and dTTP; 10 mM each in sterile double distilled water, pH 8.5), 2 μ l of oligo-p(dT)₁₅ primer (0.02 A260 units/ μ l (0.8 μ g/ μ l)), 1 μ l of RNase inhibitor (50 U/ μ l) and 0.8 μ l of AMV reverse transcriptase (25 U/ μ l) was made, to which the appropriate amount of mRNA (100 ng) was added, along with the relevant volume of molecular biology-grade H₂O, to make a total volume of 20 μ l, as shown in Table 2.6. The reaction mixture was briefly vortexed and centrifuged followed by an incubation period of 10 min at 25°C to allow the oligo-(dT)₁₅ primer to anneal to the mRNA. The reaction mixture was then further incubated for 60 min at 42°C, during which the mRNA template was reverse transcribed into single-stranded cDNA. As a final step, AMV Reverse Transcriptase was denatured by further incubating the reaction mixture for 5 min at 99°C, followed by cooling on ice for 5 min. The synthesised cDNA sample was then stored at -20°C until required.

Table 2.6 The quantities of reagents required for each cDNA synthesis reaction using those provided within the First Strand cDNA Synthesis Kit for RT-PCR (AMV).

| Reagent | Quantity |
|---|-----------------------------|
| 10 X reaction buffer | 2 μ l |
| MgCl ₂ | 4 μ l |
| Deoxynucleotide (dNTP) Mix | 2 μ l |
| Oligo-p(dT) ₁₅ primer | 2 μ l |
| RNase inhibitor | 1 μ l |
| AMV reverse transcriptase | 0.8 μ l |
| mRNA sample [†] | |
| Molecular biology-grade H ₂ O [‡] | |
| Total reaction volume | 20 μl |

[†] The volume of mRNA sample added to each reaction was determined by its concentration following extraction. Since 50–100 ng of mRNA is typically required for successful cDNA synthesis, 100 ng of extracted mRNA was added to each sample reaction.

[‡] Following the addition of 100 ng of extracted mRNA sample, the total reaction volume was made up to a final volume of 20 μ l using molecular biology-grade H₂O.

2.4 Primer Preparation

HSPA and *GAPDH* sequence-specific primers were designed using Primer3 software and commercially synthesised by TIB MOLBIOL syntheselabor (Berlin, Germany). Primers were received in a powdered form and collected by centrifugation at 10,000 g for 1 min at room temperature. Molecular biology-grade H₂O (250 µl) was added to each primer to create 20 µM stock solutions. Finally, 20 µl of the sense and antisense primer stock solutions were combined to generate a 10 µM PCR primer mix, which was then stored at -20°C until required. Table 2.10 portrays the sequences of the primers used in this study.

2.5 Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Polymerase chain reaction (PCR) is a method that allows logarithmic amplification of short DNA sequences (usually 100 to 600 bases) within longer double stranded DNA molecules. qRT-PCR allows detection of very low copies of mRNA that would otherwise not show up if other analytical techniques such as northern blotting was used. The levels of *HSPA* and *GAPDH* (reference genes used in RT-PCR reaction, Olsvik *et al*, 2005) within all the cell lines used in this study were measured by performing real-time qRT-PCR (Ball *et al*, 2003) using the LightCycler 2.0 system (Roche Diagnostics Ltd, Germany) and LightCycler[®] FastStart DNA Master^{PLUS} SYBR Green I kit, according to the manufacturer's instructions. The composition and volumes of all reagents provided are shown in Table 2.7, whilst the specific quantities used during the amplification procedure shown in Table 2.8. All samples and reagents were kept on ice throughout the procedure.

Table 2.7 The composition and quantity of each reagent provided within the LightCycler® FastStart DNA MasterPLUS SYBR Green I kit.

| Reagent | Reagent Composition | Quantity |
|--|---|---------------------------------|
| Enzyme (1a) | FastStart Taq DNA Polymerase | 1 vial |
| Reaction mix (1b) | Reaction buffer, dNTP mix (with dUTP instead of dTTP), SYBR Green I dye and MgCl ₂ | 3 vials |
| Molecular biology-grade H ₂ O | RNase-free H ₂ O | 2 ml (2 vials; 1 ml in each) |

Table 2.8 The quantities of reagents required for each RT-PCR reaction using those provided within the LightCycler® FastStart DNA MasterPLUS SYBR Green I kit.

| Reagent | Quantity |
|--|----------|
| Molecular biology-grade H ₂ O | 12 µl |
| PCR primer mix | 2 µl |
| Master Mix* | 4 µl |
| Single-stranded cDNA template | 2 µl |
| Total reaction volume | 20 µl |

* The Master Mix was formed by transferring 14 µl of enzyme (Table 2.7) into the vial of reaction mix (Table 2.7).

Prior to using hot-start PCR, an enzyme reaction mix was prepared by transferring 14 µl of LightCycler® FastStart Enzyme (1a) into the LightCycler® FastStart Reaction Mix SYBR Green vial (1b) (Table 2.8). Each reaction capillary contained a total reaction volume of 20 µl, comprising 4 µl of the ready to use hot-start reaction mix, 12 µl of molecular biology-grade H₂O, 2 µl of 10 µM PCR primer mix (generated from section 2.4 for each specific gene) and 2 µl of single stranded cDNA template. A further 20 µl of template-free reaction mix was prepared as a negative control (in which molecular biology-grade H₂O was substituted for cDNA). Prior to qRT-PCR being carried out, the definitive annealing temperature for each gene was established (Table 2.10). The LightCycler protocol for use with FastStart DNA Master^{PLUS} SYBR Green Kit 1 utilised the program, shown in Table 2.9.

Quantitative real-time PCR was used to evaluate the expression of *HSPA* (predicted amplicon size of 213 bp) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a control (predicted amplicon size of 238 bp) using FastStart DNA Master^{PLUS} SYBR Green 1 (Roche, UK). Primers used for *HSPA* were 5' CGACCTGAACAAGAGCATCA 3' (sense) and 5' AAGATCTGCGTCTGCTTGGT 3' (antisense) and for *GAPDH* primers were 5' GAGTCAAGCGATTTGGTCGT 3' (sense) and 5' TTGATTTTGGAGGGATCTCG 3' (antisense). All primers were designed using Primer3 software and manufactured by TIB MOLBIOL. The PCR protocol involved a hot start induction, with the FastStart *Taq* DNA polymerase enzyme activated by pre-incubating the reaction mixture at 95°C for 10 min. Hot start is an essential step which prevents non-specific elongations and increases PCR sensitivity, specificity and yield (Dang and Jayasena, 1996). The single stranded cDNA template was then subjected to 35 amplification cycles, each possessing the following parameters: denaturation at 95°C for 10 s, annealing at the primer dependant temperature 57°C (*HSPA*) and 56°C (*GAPDH*) for 15 s and extension at 72°C for 25 bp /

s (amplicon dependant, *HSPA* 9 s, *GAPDH* 10 s) (Patel *et al*, 2008). At the end of each cycle, the fluorescence emitted was measured in a single step in channel F1 (gain1) to obtain data for quantification analysis (Shervington *et al*, 2007b, Mohammed and Shervington, 2007). After the 35th cycle, the amplicons were prepared for melting curve analysis, heated to 95°C (denaturation) and then rapidly cooled to the previously used annealing temperature (+10°C) for 40 s. All heating and cooling steps were performed with a slope of 20°C / s. To obtain the data for the melting curve analysis, the temperature was subsequently raised to 95°C with a slope of 0.1°C / s and the emitted fluorescence was measured continuously (channel F1, gain1). Melting curve analysis was used to assess the specificity of the amplified PCR product and allow for discrimination between primer-dimers and specific product. At the final step, the generated amplicons were cooled to 40°C for 30 s and stored at -20°C until required for further analysis. All PCR reactions were performed in triplicate and a negative control included, which contained primers but no DNA.

Table 2.9 LightCycler program utilising FastStart DNA MasterPLUS SYBR Green Kit

| Cycles | Analysis Mode | Target Temperature | Hold Time | |
|---------------|---------------------------|---------------------------------------|---|--|
| 1 | None | 95°C | 10 min | Pre-incubation |
| 35 | None | 95°C | 15 s | Denaturation |
| 35 | Quantification | 57°C <i>HSPA</i> 56°C <i>GAPDH</i> | 15 s | Annealing, amplification and real time analysis |
| 35 | | 72°C | 9 sec <i>HSPA</i> 10sec <i>GAPDH</i> | Extension |
| | Melting Curve analysis | 95°C | | Denaturation |
| 1 | | 67°C <i>HSPA</i> 66°C <i>GAPDH</i> | 40 s | |
| 1 | | 95°C | | Melting |
| 1 | None | 40°C | 30 s | Cooling |

Table 2.10. The *HSPA* and *GAPDH* for *Homo sapiens* and *Rattus norvegicus* primers [designed using Primer3 software and commercially synthesised by TIB MOLBIOL syntheseslabor (Berlin, Germany)] utilised in real-time RT-PCR.

| Gene | Primer Sequences | Annealing Temperature (°C) | | | | Expected amplicon size (bp) | Extension time (s) (amplicon dependant - 25bp/s) |
|------------------------------------|--|----------------------------|----------------|----------------|--------------------------|-----------------------------|--|
| | | Primer3 | TIB MOLBIOL | GC / AT rule* | Experimental temperature | | |
| <i>HSPA Homo sapien</i> | Sense: 5' - CGACCTGAACAAGAGCATCA - 3' Antisense: 5' - AAGATCTGCGTCTGCTTGGT - 3' | 59.98 60.02 | 55.50 56.80 | 60.00 60.00 | 57 | 213 | 9 |
| <i>GAPDH Homo sapien</i> | Sense: 5' - GAGTCAACGGATTTGGTCGT - 3' Antisense: 5' - TTGATTTTGGAGGGATCTCG - 3' | 59.97 60.01 | 56.20 54.80 | 60.00 58.00 | 56 | 238 | 10 |
| <i>HSPA Rattus norvegicus</i> | Sense: 5' - GTGTGGAGAGCCAAGAGGAG - 3' Antisense: 5' - TTTCCAAACTGGATCGAAGG - 3' | 59.99 60.04 | 56.50 55.60 | 64.00 58.00 | 56 | 156 | 7 |
| <i>GAPDH Rattus norvegicus</i> | Sense: 5' - AGTGCCAGCCTCGTCTCATA - 3' Antisense: 5' - GGATCTCGCTCCTGGAAGAT - 3' | 60.97 60.70 | 57.10 56.00 | 62.00 62.00 | 57 | 265 | 11 |

* GC / AT rule: A method of calculating the primer annealing temperature using the formula: $T = 2^{\circ} (A + T) + 4^{\circ} (G + C)$, where A, C, G and T represent the number of adenine, cytosine, guanine and thymine bases respectively in the primer sequence concerned.

2.6 Analysis of qRT-PCR

2.6.1 Agarose gel electrophoresis

The amplicons from qRT-PCR were visualized on 2% agarose gels as described in section 2.2.2.

2.7 Quantification analysis of qRT-PCR

Genomic DNA of known concentrations was used as a standard to amplify *GAPDH* gene using the LightCycler instrument (Shervington *et al*, 2007b). A standard curve was produced using the crossing points shown in section 3.11.1, Figure 3.35 generated from five concentrations of genomic DNA in duplicate: 0.005, 0.05, 0.05, 5 and 50 ng with known copy numbers shown in section 3.11.1, Table 3.6. The equation generated ($y = -1.3124\ln(x) + 32.058$) was rearranged to $(=EXP ((Ct \text{ value } -32.058/-1.3124))$ and used to determine copy numbers of *HSPA* and *GAPDH* mRNA expression throughout this thesis.

2.8 Immunofluorescence

The cell culture medium was removed by gentle aspiration from the chamber slides and the cells were washed three times with warm PBS (0.1 M) before fixation. The fixation procedure involved incubation of the cells for 10 min at room temperature with freshly made 4% paraformaldehyde (w/v) in PBS (0.1 M) and 0.1 M, NaOH (sodium hydroxide). The excess paraformaldehyde was removed and the cells were washed again three times with warm PBS (0.1 M). The cells were permeabilized using 0.3 % (w/v) Triton X-100 in PBS and incubated for 7 min at room temperature on a shaker. Bovine Serum Albumin (BSA) blocking solution (1% w/v in PBS) was added to prevent

any non-specific binding and samples were incubated for 30 min at room temperature. After incubation, the blocking solution was removed by gentle aspiration. The primary antibody HSPA (Anti-Hsp70 antibody [BRM-22]) diluted in blocking solution, (dilution factor 1:200) (Abcam, UK, Cat No. ab6535) was added to each chamber and incubated at 4°C overnight. The primary antibody was removed by gentle aspiration followed by three consecutive washes with warm PBS (0.1 M). The cells were incubated with light sensitive Anti-mouse IgG FITC (fluorescein isothiocyanate) conjugated secondary antibody (Goat polyclonal Secondary Antibody to Mouse IgG - H&L FITC, Abcam, UK, Cat. No. ab6785) diluted in blocking solution (dilution factor 1:128) for a further 60 min at room temperature on a shaker in a dark room. The secondary antibody was removed by gentle aspiration, followed by three consecutive washes with warm PBS (0.1 M). The sections were mounted under a cover slip using VECTASHIELD PI (0.01 M) (Propidium Iodide, Vector, USA) mounting medium. The cells were then visualized and scanned using an Axiovert 200 LSM 510 laser scanning confocal microscope (Carl Zeiss, USA). Negative control cells from each sample underwent identical preparations for immunofluorescence staining, except that the primary antibody was omitted. Routinely 500 cells were analysed per sample.

2.9 Flow Cytometry

Cells were washed once in 0.1% BSA in PBS (500 µl), centrifuged for 5 min at 1000 rpm, and the supernatant was removed. Triton X-100 0.1% (100 µl) was added and samples incubated on ice for 15 min. Following incubation, the suspension was centrifuged for 5 min at 1000 rpm, and the supernatant was removed. Cells were washed once in 0.1% BSA in PBS (500 µl) and samples centrifuged for 5 min at 1000 rpm, after which the supernatant was removed. Goat serum (5% in PBS) and 0.1% BSA (50 µl) was added to each sample, which were then incubated on ice for 30 min. Following incubation, the suspension was centrifuged for 5 min at 1000 rpm, and the

supernatant was removed. Primary antibody (HSPA) (Anti-Hsp70 antibody [BRM-22], Abcam,UK) in PBS, 0.1% BSA and goat serum 5% (50 μ l) was added and incubated on ice for 30 min. Following incubation, the suspension was centrifuged for 5 min at 1000 rpm, before the supernatant was removed. Cells were washed once in 0.1% BSA in PBS (500 μ l) and centrifuged for 5 min at 1000 rpm, and the supernatant was removed. Fluorescein isothiocyanate (FITC) conjugated secondary antibody (Goat polyclonal Secondary Antibody to Mouse IgG - H&L FITC, Abcam, UK,) in PBS, 0.1% BSA and goat serum 5% (50 μ l) was added to each sample and they were then incubated on ice for 30 min. Following incubation, the suspension was centrifuged for 5 min at 1000 rpm, and the supernatant removed. Cells were washed twice in 0.1% BSA in PBS (500 μ l) and centrifuged for 5 min at 1000 rpm, and the supernatant was again removed. Cells were resuspended in 0.1% BSA in PBS (300 μ l) and filtered into FACS tubes. Becton Dickinson FACSAria flow cytometry equipment was used to count and distinguish between cells for the presence or absence of HSPA.

2.10 *Rattus norvegicus* Brain Tissue

Studies were undertaken using post-mortem brain tissue samples from aged-matched male Wistar rats (obtained from the Physiology Laboratory, University of Central Lancashire). All procedures conformed to the –UK Animals (Scientific Procedures) Act 1986 according to the –Principles of Laboratory Animal Care, 1985. The work had the relevant ethical clearance from the Ethics Committee for the University of Central Lancashire under Home Office Licence PIL50/00824. Animals were housed in groups under institutional regulations at standard vivarium conditions, granted free access to water and commercial chow, exposed to a 12 h light / 12 h dark cycle and monitored for any signs of stress. Rats were humanely killed by cervical dislocation and the brain removed, either immediately or at the given post-mortem interval.

Brain tissue samples weighing approximately 50 mg – 100 mg and no less than 0.5 cm in thickness, were excised using a sterile scalpel at timed intervals of 0, 3, 6, 12 and 24 h and maintained at room temperature. Excised samples after sacrifice were immediately weighed and snap-frozen by immersion into liquid nitrogen, followed by grinding of the tissue to a fine powder using a pestle and mortar under liquid nitrogen. Samples were transferred to new sterile 1.5 ml RNase free microfuge tubes and stored at -80°C.

2.11 Treatment

2.11.1 Induction of mild heat shock

Additional brain tissue excised at 0, 3, 6, 12 and 24 h post sacrifice was subjected to mild heat shock. This was performed by placing the brain tissue into sterile 1.5 microfuge tubes and immersion in a heated water bath at 42°C for a period of 10 min, after which samples were stored at room temperature. Samples weighing approximately 30 mg – 50 mg, and no greater than 0.5 cm in thickness, were excised using a sterile scalpel at timed intervals of 0, 3, 6, 12 and 24 h post sacrifice. Excised samples were immediately weighed and snap-frozen, by immersion in liquid nitrogen followed by grinding of the tissue to a fine powder using a sterile pestle and mortar, again under liquid nitrogen, before transfer to sterile 1.5 ml RNase free microfuge tubes and storage at -80°C.

2.11.2 Induction of multiple heat shocks

Additional rat brain tissue excised at 0, 3, 6, and 24 h post sacrifice was subjected to mild heat shock at 0 h and again at 3, 6 and 24 h time course periods. This was performed by placing the brain tissue into sterile 1.5 microfuge tubes and immersion in a heated water bath at 42°C for a period of 10 min, after which samples were stored at room temperature. Samples weighing approximately 30 mg – 50 mg, and no greater than 0.5 cm in thickness, were excised using a sterile scalpel at timed intervals of 0, 3, 6, and 24 h post sacrifice. Excised samples were immediately weighed and snap-frozen, by immersion in liquid nitrogen followed by grinding of the tissue to a fine powder using a sterile pestle and mortar, again under liquid nitrogen, before transfer to sterile 1.5 ml RNase free microfuge tubes and storage at -80°C.

2.12 DNA Extraction

DNA was extracted from brain tissue using DNeasy[®] Blood and Tissue Kit (Qiagen, UK. Cat. No. 69504) and RNase A (Qiagen, UK. Cat. No. 19101). Prior to commencing this procedure, 25 ml and 30 ml ethanol (96 -100%) was added to 95 ml of AW1 and 66 ml of AW2 buffer concentrates respectively.

Table 2.11. Reagents, components and quantity provided of each in the DNeasy[®] Blood and Tissue Kit (Qiagen).

| Reagent / Component | Quantity |
|---|---|
| DNeasy Mini Spin Columns in 2 ml Collection Tubes | 50 |
| Collection Tubes 2 ml | 100 |
| Buffer ATL | 10 ml |
| Buffer AL | 12 ml |
| Buffer AW1 (concentrate) | 19 ml |
| Buffer AW2 (concentrate) | 13 ml |
| Buffer AE | 22 ml |
| Proteinase K | 1.25 ml |
| RNase A (17,500 U) | 2.5 ml (100mg/ml; 7000 units/ml solution) |

DNA was isolated from 25 mg of ground brain tissue following the manufacturer's protocol. The volumes of each reagent and buffers provided within the kit are detailed in Table 2.11, whilst the specific measures used during each extraction procedure are

shown in Table 2.12. Initially, 180 µl of Buffer ATL was added to 25 mg of ground brain tissue with 20 µl of Proteinase K. Samples were vortexed thoroughly and incubated overnight at 56°C. Following overnight incubation, 4 µl of RNase A (100 mg/ml) was added to each sample and mixed by vortexing before incubating for 2 min at room temperature. After incubation, the lysed samples were vortexed for 15 s and 200 µl of Buffer AL was added and mixed thoroughly by vortexing, followed by the addition of 200 µl of ethanol (100%) which was again mixed thoroughly by vortexing. The lysis solution was pipetted into a DNeasy mini-spin column placed in a 2 ml collection tube and centrifuged at 6000 g (8000 rpm) for 1 min. The flow-through and the collection tube were discarded. The DNeasy mini-spin column was placed in a new 2 ml collection tube, 500 µl of Buffer AW1 was added and centrifuged at 6000 g (8000 rpm) for 1 min. The flow-through and the collection tube were again discarded. The DNeasy mini-spin column was placed in a new 2 ml collection tube 500 µl of Buffer AW2 was added and centrifuged at 20000 g (14000 rpm) for 3 min, before the flow-through and the collection tube were discarded. The DNeasy mini-spin column was placed in a new 1.5 ml micro-centrifuge tube and 200 µl of Buffer AE was added directly onto the DNeasy membrane, incubated at room temperature for 1 min and centrifuged at 6000 g (8000 rpm) for 1 min. The resulting supernatant (DNA) was stored at -20°C ready for quantification and analysis.

Table 2.12. Volumes of reagents used for DNA extraction as per manufacturers' protocol.

| Amount of Tissue | 25 mg |
|--------------------------|--------------|
| Buffer ATL | 180 µl |
| Buffer AL | 200 µl |
| Buffer AW1 (concentrate) | 500 µl |
| Buffer AW2 (concentrate) | 500 µl |
| Buffer AE | 200 µl |
| Proteinase K | 20 µl |
| RNase A (17,500 U) | 4 µl |
| Ethanol 100% | 200 µl |

2.12.1 Quantification of nucleic acids

Extracted DNA was quantified by measurement of absorbance of samples using the Thermo Scientific NanoDrop™ 1000 spectrophotometer at wavelengths of 260 nm and 280 nm. A 2 µl DNA sample was pipetted onto the end of a fiber optic cable (the receiving fiber). A second fiber optic cable (the source fiber) was then brought into contact with the liquid sample, causing the liquid to bridge the gap between the fiber optic ends. A pulsed xenon flash lamp provide the light source and a spectrometer utilizing a linear CCD array was used to analyse the light passing through the sample (<http://www.nanodrop.com/Library/nd-1000-v3.7-users-manual-8.5x11.pdf>). The ratio of 260 nm to 280 nm was used to determine the purity of the extracted DNA. A ratio of ~ 1.8 was generally accepted as indicative of pure DNA.

2.12.2. Analysis of Nucleic acid by agarose gel

Extracted DNA was visualized on 2% agarose gels to determine whether DNA was intact or degraded as per section 2.2.2.

2.12.3. Quantification analysis of qRT-PCR

Extracted *Rattus norvegicus* genomic DNA was used as a standard to amplify *GAPDH* gene using the LightCycler instrument. A standard curve was produced using the crossing points (section 3.11.1, Figures 3.33) generated from five concentrations of genomic DNA in duplicate: 0.005, 0.05, 0.05, 5 and 50 ng with known copy numbers (section 3.11.1, Table 3.5). The equation generated ($y = -1.3124\ln(x) + 32.058$) was rearranged to $(=EXP ((Ct \text{ value } -32.058/-1.3124))$ and used to determine copy numbers of *HSPA* and *GAPDH* mRNA expression throughout this thesis.

2.14 mRNA Isolation

mRNA was isolated using mRNA Isolation Kit (Roche-applied-science, Germany Cat. No. 11 741 985 001). Reagents were mixed using a vortex mixer and briefly centrifuged prior to commencing this procedure.

mRNA was isolated from 50 mg of ground brain tissue following the manufacturer's protocol as shown in the schematic diagram Figure 2.1. The volumes and composition of each reagent and buffers provided within the kit are detailed in Table 2.3, whilst the specific measures used during each extraction procedure are shown in Table 2.4.

Initially, 1.5 ml of lysis buffer (0.1 M Tris buffer, 0.3 M LiCl, 10 mM EDTA, 1% (w/v) lithium dodecylsulphate, 5 mM DTT (dithiothreitol) pH 7.5) was chilled to $\leq 0^{\circ}\text{C}$ in a

sodium chloride-ice water bath, after which 50 mg of ground brain tissue was added followed by mechanical shearing of tissue achieved by passing through a 21 gauge needle (four). The suspension was centrifuged at 11,000 g for 30 s between 0°C and -4°C. The supernatant was captured and transferred into a 1.5 ml sterile microfuge tube and placed on ice. An aliquot of 1.5 µl of biotin labelled oligo(dT)₂₀ probe (100 pmol biotin-labelled oligo(dT)₂₀ per µl of molecular biology grade H₂O) was added to the tissue lysate and allowed to hybridise with mRNA for 10 min at room temperature. Simultaneously, 150 µl of streptavidin magnetic particles (10mg/ml of suspension in 50 mM Hepes, 0.1 % (w/v) bovine serum albumin, 0.1 % (w/v) chlorace tamide, 0.01 % (w/v) methylisothiazolone, pH 7.4) was aliquoted into a 1.5 ml sterile microfuge tube and separated from the storage buffer by placing in a magnetic separator, after which the storage buffer was discarded. The particles were cleansed by re-suspension in 250 µl of lysing buffer (0.1 M Tris buffer, 0.3 M LiCl, 10 mM EDTA, 1% (w/v) lithium dodecylsulphate, 5 mM (dithiothreitol) DTT pH 7.5). Following magnetic separation and disposal of the supernatant, the prepared particles were resuspended in the dT-mRNA hybrid mixture. After briefly vortexing, the resultant suspension was incubated for 5 min at 0°C to achieve immobilisation. Following incubation, the hybrid-linked particles were magnetically separated from this fluid and the supernatant was discarded. The remaining particles were then washed by resuspension in 3 × 200 µl of washing buffer (10 mM Tris buffer, 0.2 M LiCl and 1 mM EDTA, pH 7.5). Upon disposal of the final supernatant, mRNA was eluted from the particles by resuspension in 10 µl of redistilled water, mixed and incubated for 2 min at 65°C. After magnetically separating the particles from the fluid, the resulting supernatant (mRNA) was stored at -20°C in an RNase free microfuge tube ready for quantification and analysis.

2.15 Complimentary DNA Synthesis (cDNA)

mRNA was reversed transcribed using First Strand cDNA Synthesis Kit harnessing AMV enzymes as per section 2.3. The composition and volumes of all reagents provided are shown in Table 2.5, whilst the specific quantities required by each cDNA synthesis reaction are shown in Table 2.6. Reagents were mixed using a vortex mixer and briefly centrifuged prior to commencing this procedure.

2.16 Primer Preparation

Rattus norvegicus GAPDH and *HSPA* sequence-specific primers were designed using Primer3 software and commercially synthesised by TIB MOLBIOL syntheselabor (Berlin, Germany). Primers were received in a powdered form and collected by micro-centrifugation at 10,000 g for 1 min at room temperature. Molecular biology-grade H₂O (250 µl) was added to each primer to create 20 µM stock solutions. Finally, 20 µl of the sense and antisense primer stock solutions were combined to generate a 10 µM PCR primer mix, which was then stored at -20°C until required. Table 2.10 portrays the sequences of the primers corresponding to each gene.

2.17 Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The levels of *HSPA* and *GAPDH* RNA sequences (*GAPDH* reference gene used in RT-PCR reaction, Olsvik *et al*, 2005) for all tissue samples were measured by performing real-time qRT-PCR (Ball *et al*, 2003) using the LightCycler 2.0 system (Roche Diagnostics Ltd, Germany) and LightCycler® FastStart DNA Master^{PLUS} SYBR Green I kit according to the manufacturer's instructions. The composition and volumes of all reagents provided are shown in Table 2.7, whilst the specific quantities used during the

amplification procedure shown in Table 2.8. All samples and reagents were kept on ice throughout the procedure.

Prior to using the hot-start PCR an enzyme reaction mix was prepared by transferring 14 µl of LightCycler® FastStart Enzyme (1a) into the LightCycler® FastStart Reaction Mix SYBR Green vial (1b) (Table 2.8). Each reaction capillary contained a total reaction volume of 20 µl, comprising 4 µl of the ready to use hot-start reaction mix, 12 µl of molecular biology-grade H₂O, 2 µl of 10 µM PCR primer mix (generated from section 2.4 for each specific gene) and 2 µl of single stranded cDNA template. A further 20 µl of template-free reaction mix was prepared as a negative control (in which molecular biology-grade H₂O substituted for cDNA). Prior to qRT-PCR being carried out, the definitive annealing temperature for each gene was established (Table 2.10). LightCycler protocol for use with FastStart DNA Master^{PLUS} SYBR Green Kit 1 utilised the program shown below in Table 3.13.

Quantitative real-time PCR was used to evaluate the expression of *HSPA* (predicted amplicon size of 156 bp) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (predicted amplicon size of 207 bp) using FastStart DNA Master^{PLUS} SYBR Green 1 (Roche, UK). Primers used for *HSPA* were 5′ GTGTGGAGAGCCAAGAGGAG 3′ (sense) and 5′ TTTCCAAACTGGATCGAAGG 3′ (antisense) and for *GAPDH* primers were 5′ AGACAGCCGCATCTTCTTGT 3′ (sense) and 5′ CTTGCCGTGGGTAGAGTCAT 3′ (antisense). All primers were designed using Primer3 software and manufactured by TIB MOLBIOL. The PCR protocol involved a hot start induction, with the FastStart *Taq* DNA polymerase enzyme activated by pre-incubating the reaction mixture at 95°C for 10 min. Hot start includes a step which prevents non-specific elongations and increases PCR sensitivity, specificity and yield (Dang and Jayasena, 1996). The single stranded cDNA template was then subjected to

35 amplification cycles, each with the following parameters: denaturation at 95°C for 10 s, annealing at the primer dependant temperature 56°C (*HSPA*) and 57°C (*GAPDH*) for 15 s and extension at 72°C for 25 bp / s (amplicon dependant, *HSPA* 7 s, *GAPDH* 9 s) (Patel *et al*, 2008). At the end of each cycle, the fluorescence emitted was measured in a single step in channel F1 (gain1) to obtain data for quantification analysis (Shervington et al, 2007b, Mohammed and Shervington, 2007). After the 35th cycle, the amplicons were prepared for melting curve analysis, heated to 95°C (denaturation) and then rapidly cooled to the previously used annealing temperature (+10°C) for 40 s. All heating and cooling steps were performed with a slope of 20°C / s. To obtain the data for the melting curve analysis, the temperature was subsequently raised to 95°C (melting) with a slope of 0.1°C / s and the emitted fluorescence was measured continuously (channel F1, gain1). Melting curve analysis was used to assess the specificity of the amplified PCR product and allow for discrimination between primer-dimers and specific product. At the final step, the generated amplicons were cooled to 40°C for 30 s and stored at -20°C until required for further analysis. All PCR reactions were performed in triplicate with a negative control included, which contained primers but no DNA.

Table 2.13 LightCycler program utilising FastStart DNA MasterPLUS SYBR Green Kit

| Cycles | Analysis Mode | Target Temperature | Hold Time | |
|---------------|------------------------|---------------------------------------|-------------------------------------|---|
| 1 | None | 95°C | 10 min | Pre-incubation |
| 35 | None | 95°C | 15 s | Denaturation |
| 35 | Quantification | 56°C <i>HSPA</i> 57°C <i>GAPDH</i> | 15 s | Annealing, amplification and real time analysis |
| 35 | | 72°C | 7 s <i>HSPA</i> 9 s <i>GAPDH</i> | Extension |
| | Melting Curve analysis | 95°C | | Denaturation |
| 1 | | 66°C <i>HSPA</i> 67°C <i>GAPDH</i> | 40 s | |
| 1 | | 95°C | | Melting |
| 1 | None | 40°C | 30 s | Cooling |

2.18 Quantification analysis of qRT-PCR

Genomic DNA of known concentrations was used as a standard to amplify *GAPDH* gene using the LightCycler instrument. A standard curve was produced using the crossing points as shown in section 3.11.1, Figure 3.33 generated from five concentrations of genomic DNA in duplicate: 0.005, 0.05, 0.05, 5 and 50 ng with known copy numbers shown in section 3.11.1, Table 3.6. The equation generated ($y = -1.3124\ln(x) + 32.058$) was rearranged to $(=EXP((Ct\ value - 32.058)/-1.3124))$ and used to determine copy numbers of *HSPA* and *GAPDH* mRNA expression throughout this thesis.

2.19 Cryostat

Frozen sections of brain tissue were placed onto a chuck containing OCT (optimal cutting temperature) compound (10.24% polyvinyl alcohol, 4.26% polyethylene glycol, 85.5% non-reactive ingredient) and placed in liquid nitrogen. Once the OCT compound had turned solid and white, the frozen samples on the chuck were placed in the Bright's cryostat for cutting. The samples were coarsely trimmed until a full flat face was visible. Sections were then cut at 10 μ (microns) thickness and carefully placed on a charged slide. A few drops of 5% acetic acid was added to the cut sections and allowed to fix for a few seconds. Slides were kept at -20°C until required for immunofluorescent experiments.

2.20 Immunofluorescence

The tissue sections were initially fixed in freshly made 4% Paraformaldehyde (w/v) in PBS (0.1 M) for 15 min at room temperature, after which the excess paraformaldehyde was removed. The fixed tissue sections were permeabilized using Trypsin (0.025%) in

aqueous calcium chloride (CaCl_2) (0.1%, pH 7.8), incubated for 45 min at 37°C, and then washed three times in warm PBS (0.1M). The sections were then incubated in blocking solution (0.1% PBS, 0.5% Tween 20, 0.1% Goat serum) for 30 min at room temperature, followed by overnight incubation at 4°C in the primary antibody for HSPA (Anti-Hsp70 antibody [BRM-22], Abcam, UK, dilution 1:200) to allow the antibody to bind, and for saturation to take place. After overnight incubation, the sections were washed with PBS three times for 5 min and incubated in a solution of a light sensitive Anti-mouse IgG FITC (Fluorescein Isothiocyanate) conjugated secondary detection antibody (Goat polyclonal Secondary Antibody to Mouse IgG - H&L FITC, Abcam, UK, dilution 1:200) diluted in blocking solution for 1 h at room temperature. The secondary antibody was removed followed by three consecutive washes with warm PBS (0.1 M). The sections were mounted under a cover slip using VECTASHIELD PI (0.01 M) (Propidium Iodide, Vector, USA) mounting medium. The tissue sections were then examined and images recorded using an Axiovert 200 LSM 510 laser scanning confocal microscope (Carl Zeiss, USA). Negative control cells from each sample encountered identical preparations for immunofluorescence staining, except that the primary antibody was omitted.

2.21 Statistical Analysis

For analysis of *HSPA*, *GAPDH* mRNA copy number and protein levels, all quantitative data was presented as the mean \pm SD of three separate experiments and subjected to either a one-way ANOVA between groups of analysis of variance test, a two-way factorial mixed ANOVA analysis of variance test, a three-way factorial mixed ANOVA analysis of variance or an independent-samples t-test using IBM SPSS statistics 19 software.

Chapter 3

Developmental Work

3.1 Developmental Work

During the course of this programme of research, initial developmental work was required to gain experience and establish the validity of the techniques and primer sequences for use in PCR experimentation. This chapter will focus on these areas of developmental work, to enable subsequent results chapters to focus on the results obtained. It will also describe the biological samples used for experimental work, and standard methodologies used.

3.2 Tissue samples and cell lines

Work undertaken throughout this study involved the use of the following samples:

- a) Tumour and normal (control) brain tissue samples were obtained from patients admitted to the Royal Preston Hospital, UK (section 4.2.3, Table 4.5). Work on these samples was carried out with ethical approval from the North Manchester Research Ethics Committee (Ref: 06/Q1406/104) and the Ethics Committee at the University of Central Lancashire.

- b) Human brain cell line grade I astrocytoma, 1321N1 was obtained from European Collection of Cell Cultures, ECACC, (UK), grade II/III oligodendroglioma GOS-3 from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DMSZ, (Germany), grade IV glioblastoma, U87-MG from ECACC (UK) and normal human astrocytes, NHA from Lonza (UK) (section 2.1.2, Table 2.1).

- c) Post-mortem brain tissue samples were obtained from aged-matched male Wistar rats from the Physiology Laboratory, University of Central Lancashire.

Due to difficulties in obtaining post-mortem human brain tissue for all experiments, rat brain tissue was chosen for some experiments in this thesis. These samples were readily accessible from the Physiology Laboratory, University of Central Lancashire under the Home Office Licence PIL50/00824. Sequence homology between *Homo sapiens* and *Rattus norvegicus* for the target genes used indicated 73% homology for *HSPA/HSP70* nucleotide sequences, 96% homology for *HSPA/HSP70* amino acid sequences, 84% homology for the control *GAPDH* nucleotide sequence and 93% homology for *GAPDH* amino acid sequence (Table 3.1).

3.3 Introduction and history of bioinformatics

Bioinformatics is an essential computational tool in molecular biology, enabling the storage, retrieval, analysis and distribution of biological data (Kamel 2003; Kim, 2000; Singh and Kumar, 2001). It is defined as –the field of science in which biology, computer science and information technology merge into a single discipline (National Centre for Biotechnology Information, NCBI). Broadly speaking, this discipline consists of three main areas:

- the development of new algorithms and statistics in order to determine relationships amongst members of large data sets,
- the analysis and interpretation of various types of data, such as nucleotide and amino acid sequences, protein domains and structures, and

- the development of tools that enable the efficient access and management of different types of information.

To date, bioinformatics encompasses a wide variety of biological data, such as protein structures, gene and protein functional data, metabolic pathways and genomes. Due to the huge amount of data involved, much work has been concerned with the development of databases such as GenBank (Genetic Sequence Databank, USA), EMBL Nucleotide Sequence Database (Europe), DDBJ (DNA Database Japan), SwissProt (Switzerland), and with the associated software required for the analysis of the stored sequence information, primarily DNA, RNA and protein sequence data from the human and other genome sequencing projects (Fenstermacher, 2005). The management and accessibility of this data has been directly attributable to the development of the World Wide Web (WWW), which has facilitated access to this data by researchers, regardless of country or in-house research facilities.

3.4 Candidate Genes

Heat shock proteins are a family of highly conserved ubiquitous proteins encoded by genes which are activated not only in response to various physiological and environmental stress conditions, but also in cells affected by various diseases, such as cancer, Alzheimer's, Parkinson's and Huntington's diseases and diabetes, or by fever or inflammation (Jolly and Morimoto, 2000; Lindquist, 1986; Morimoto, 1993). However, in contrast to the diversity of the heat shock protein families, which cover approximately five hundred client proteins, the present study will only focus on HSPA/HSP70.

The *HSPA/HSP70* gene family is one of the most predominant heat shock protein gene families and is of particular interest due to three well-characterized members; *HSPA1A/HSP70-1*, *HSPA1B/HSP70-2* and *HSPA1L/HSP70-Hom*; (Milner and Campbell, 1990). Genes for these members are located within the major histocompatibility complex class III region (MHC-III), for *Homo sapiens* located on the short arm of chromosome 6 (6p21.3), and are intronless and for *Rattus norvegicus* located on the short arm of chromosome 20 (20p12), and again are intronless.

Although these three genes have similar nucleotide sequences, they differ in their regulation. Both *HSPA1A* and *HSPA1B* have been shown to encode identical protein products of 641 amino acids, and *HSPA1L* has been identified at a location 4 kb telomeric to *HSPA1A* with a 90% similarity in amino acid sequence (Milner and Campbell, 1990; Sargent *et al*, 1989a; Sargent *et al*, 1989). Both *HSPA1A* and *HSPA1B* have been shown to be highly expressed in mammalian cells that have been subjected to heat shock at 42°C (Milner and Campbell, 1990). *HSPA1A* is also constitutively expressed at low levels (Milner and Campbell, 1990). *HSPA1L*, which has no associated regulatory heat shock consensus sequence, has been shown to be expressed at low levels both constitutively and after heat shock at 42°C, with no comparable difference in levels (Milner and Campbell, 1990).

GAPDH was chosen as the preferred housekeeping gene to be used throughout this study. *GAPDH* is ubiquitously expressed in cells and has been shown to remain relatively constant in RNA and protein levels in normal and tumourous brain tissue (Barber *et al*., 2005; Said *et al*, 2007).

3.6 Nucleotide sequences

Nucleotide sequences for those genes of interest in this study were located using public databases, such as GenBank, SwissProt and EMBL which are held by the National Centre for Biotechnology Information (NCBI, Unigene). The NCBI database contains a collection of Expression Sequence Tags (ESTs), small sub-sequences derived from cDNA sequences (approx. 200 – 500 bp in length) that are generated by sequencing either one or both ends of RNA transcripts. ESTs are mapped to corresponding genomes, providing information regarding putative genes that have been located through analysis of biocomputational techniques.

The mRNA sequences used for this study were accessed from NCBI at:

<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide&cmd=search&term>

The sequences obtained are shown in Appendix 9.1 (*HSPA1A Homo sapiens*), 9.2 (*HSPA1B Homo sapiens*), 9.3 (*HSPA1L Homo sapiens*), 9.4 (*HSPA1A Rattus norvegicus*), 9.5 (*HSPA1B Rattus norvegicus*), 9.6 (*HSPA1L Rattus norvegicus*), 9.7 (*GAPDH Homo sapiens*) and 9.8 (*GAPDH Rattus norvegicus*).

3.7 Primer Design

3.7.1 Primer Specificity

One of the most critical parameters for successful PCR is the design of amplification primers (Kamel *et al*, 2003). The efficacy, specificity and sensitivity of PCR depend largely on the efficiency of these primers (Dieffenbach *et al*, 1993; He *et al*, 1994). The sequence of each of the designed primers in a pair determines their specificity, melting temperature, G/C content and product length. There are numerous bioinformatic software programs available, such as primer-Blast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>) and Primer 3 (<http://frodo.wi.mit.edu/primer3/>), which assist in the design of effective PCR primers from a template DNA sequence (Dieffenbach *et al*, 1993; Kamel *et al*, 2003).

3.7.2 Primer Length

For RT-PCR, the most efficient primers are between 18 and 24 nucleotides in length (Dieffenbach *et al*, 1993; He *et al*, 1994; Kamel *et al*, 2003). In general, primers between 18 and 24 nucleotides in length tend to be very specific, particularly if the annealing temperature is set within a few degrees of the primer melting temperature (Dieffenbach *et al*, 1993). Depending on the size of the genome for the organism being studied, there is a minimum optimal primer length. For each additional nucleotide, the primer becomes four times more specific and hence the minimum primer length used in most applications is 18 nucleotides (Dieffenbach *et al*, 1993). Primers longer than 24 nucleotides in length may have a tendency to form secondary structures, which can

result in primer dimer formation and hence decrease PCR efficiency (Dieffenbach *et al*, 1993; He *et al*, 1994; Kamel *et al*, 2003).

3.7.3 Primer Melting (Annealing) Temperature (T_m)

Primer melting temperature (T_m) is important in determining the optimal PCR annealing temperature, which is in turn determined by primer sequence, primer concentration, salt concentration and magnesium chloride (MgCl₂) concentration. Ideally, both forward and reverse primers should have similar optimal melting temperatures, within the range of 52 – 62°C (Dieffenbach *et al*, 1993; He *et al*, 1994; Kamel *et al*, 2003). As a rule of thumb, most primer design programs use the following 4 + 2 rule, based purely on primer GC content, to calculate the optimal primer melting temperature:

$$T_m = 4 * (G + C) + 2 * (A + T)$$

The T_m is calculated by counting the numbers of guanine or cytosine residues and adenine or thymine residues in both the forward and reverse primers and substituting this information in the above equation. Each G/C effectively represents 4°C and A/T represents 2°C of the calculated melting temperature.

3.7.4 Primer GC content

For most PCR applications, the primer G/C content should be within the range of 45 – 60% of the total sequence (Dieffenbach *et al*, 1993; Kamel *et al*, 2003). If the G/C content is too low, then this could result in decreased PCR efficiency and poor primer

binding. Alternatively, if the G/C content is too high, this could result in mispriming through the formation of a stably annealed duplex with non-target templates (Dieffenbach *et al*, 1993; Kamel *et al*, 2003).

3.7.5 Product Length (Amplicon Size)

For accurate quantification and PCR efficiency, the product length should be within the range of 100 – 250 bp. Products longer than 250 bp will lead to decreased PCR efficiency (Dieffenbach *et al*, 1993; Kamel *et al*, 2003). The primers were designed using Primer 3 software, accessible at the website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to produce amplicons in this size range.

3.7.6 Experimental design of Primers

All primers were ordered and commercially synthesised by TIB MOLBIOL syntheselabor (Berlin, Germany). All primers were rehydrated in molecular biology grade H₂O (250 µl) to create 20 µM stock solutions which were stored at -20°C until required. Primers were designed using Primer 3 software using the following design parameters: primer length 20 ± 2 bp; G/C content between 45 – 60%; primer melting temperature between 52 – 62°C; and avoidance of the GC-rich 3' end (4 or more G's or C's in a row). Any potential hairpin formation, (i.e. self-complementarity of primers) was checked using the oligonucleotides properties calculator (<http://www.basic.northwestern.edu/biotools/oligocalc.html>). Section 2.7, Table 2.10 portrays the sequences of the primers used in this study.

Nucleotide sequences obtained from NCBI for *HSPA1A*, *HSPA1B*, *HSPA1L* and *GAPDH*, for both *Homo sapiens* and *Rattus norvegicus*, (appendices 9.1 – 9.8) were copied and pasted into a software program Primer3, which designs primers required for real time qRT-PCR (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The program generated a number of possible forward and reverse primers, identifying primer location and length, amplicon size, GC % and suggested annealing temperature. The primers selected for this study were chosen based on the above parameters. Product lengths were between 156 – 238 bp, G/C content was between 45 – 60%, melting temperatures was between 59 – 61°C, primers contained no GC-rich 3' ends and did not contain any hairpin formations (appendices 9.9 – 9.13). A Basic Local Alignment Search Tool (BLAST) analysis was performed to find regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches (NCBI) (Appendix xx).

3.8 Sequence Homology for HSP70 between *Homo sapiens* and *Rattus norvegicus*

General purpose multiple sequence alignment programs were used to determine sequence homology between the human and rat genes used in this study. This provides information useful in identifying conserved sequence regions and can also show multiple alignments for nucleic acid and protein sequences between species. Alignment of sequences can be carried out either along the entire length (global alignment) or restricted to certain regions (local alignment); (Kulikova *et al*, 2004) European Bioinformatics Institute (EBI) (<http://www.ebi.ac.uk>). The best matches for selected sequences are calculated and aligned so that identities, similarities and differences may be observed (Chenna *et al*, 2003).

The nucleotide and amino acid sequences obtained from NCBI for *HSPA1A*, *HSPA1B*, *HSPA1L* and *GAPDH* for both *Homo sapiens* and *Rattus norvegicus* (appendices 9.1 to 9.8) were copied and pasted into the software programme ClustalW2 <http://www.ebi.ac.uk/clustalw/>. Results for gene sequence and amino acid sequence homology by species and between species are shown in appendices 9.14 -9.25 and Table 3.1.

For *Homo sapiens*, results indicated 90%, 64% and 61% homology for *HSPA1A* v *HSPA1B*, *HSPA1A* v *HSPA1L* and *HSPA1B* v *HSPA1L* nucleotide sequences respectively, and 100%, 90% and 89% homology for amino acid sequences respectively. For *Rattus norvegicus*, results indicated 78%, 73% and 74% homology for *HSPA1A* v *HSPA1B*, *HSPA1A* v *HSPA1L* and *HSPA1B* v *HSPA1L* nucleotide sequences respectively, and 100%, 80% and 80% homology for amino acid sequences respectively. Sequence homology between *Homo sapiens* and *Rattus norvegicus* indicated 73%, 78%, 78% and 84% homology for *HSPA1A*, *HSPA1B*, *HSPA1L* and *GAPDH* nucleotide sequences respectively and 96%, 96%, 94% and 93% homology for *HSPA1A*, *HSPA1B*, *HSPA1L* and *GAPDH* amino acid sequences respectively.

Table 3.1. Table showing gene and amino acid sequence homology between genes and species.

| Species | Gene | Gene Sequence Homology % | Amino Acid Sequence Homology % |
|--|------------------------|--------------------------|--------------------------------|
| <i>Homo sapiens</i> | | | |
| | <i>HSPA1A v HSPA1B</i> | 90 | 100 |
| | <i>HSPA1A v HSPA1L</i> | 64 | 90 |
| | <i>HSPA1B v HSPA1L</i> | 61 | 89 |
| <i>Rattus norvegicus</i> | | | |
| | <i>HSPA1A v HSPA1B</i> | 78 | 100 |
| | <i>HSPA1A v HSPA1L</i> | 73 | 80 |
| | <i>HSPA1B v HSPA1L</i> | 74 | 80 |
| <i>Homo sapien v Rattus norvegicus</i> | | | |
| | <i>HSPA1A</i> | 73 | 96 |
| | <i>HSPA1B</i> | 78 | 96 |
| | <i>HSPA1L</i> | 78 | 94 |
| | <i>GAPDH</i> | 84 | 93 |

Results from Clustal W2 indicated for *Homo sapiens*, 90%, 64% and 61% homology for *HSPA1A v HSPA1B*, *HSPA1A v HSPA1L* and *HSPA1B v HSPA1L* nucleotide sequences and 100%, 90% and 89% homology for amino acid sequences respectively. For *Rattus norvegicus*, results indicated 78%, 73% and 74% homology for *HSPA1A v HSPA1B*, *HSPA1A v HSPA1L* and *HSPA1B v HSPA1L* nucleotide sequences and 100%, 80% and 80% homology for amino acid sequences respectively. Results between species indicated 73%, 78%, 78% and 84% homology for *HSPA1A*, *HSPA1B*, *HSPA1L* and *GAPDH* nucleotide sequences and 96%, 96%, 94% and 93% homology for *HSPA1A*, *HSPA1B*, *HSPA1L* and *GAPDH* amino acid sequences respectively.

3.9 Spectrophotometry

Spectrophotometry was conducted on the extracted mRNA samples obtained from each cell line and from rat brain tissue samples, to establish the purity and yield of nucleic acids. The absorbance of samples was measured at wavelengths of 260 and 280 nm, and the concentration of mRNA present in each cell line was calculated as described in Section 2.2.1. Examples of typical results obtained from mRNA samples are presented below in Table 3.2.

Table 3.2 An example of the obtained spectrophotometric readings and the subsequent concentrations of the mRNA isolated from all four cell lines used in this study.

| Cell line | A ₂₆₀ reading | A ₂₈₀ reading | A ₂₆₀ / A ₂₈₀ ratio | Concentration (µg/ml) |
|-----------|--------------------------|--------------------------|---|-----------------------|
| 1321N1 | 0.010 | 0.005 | 2.000 | 100 |
| U87- MG | 0.013 | 0.007 | 1.857 | 130 |
| GOS-3 | 0.062 | 0.032 | 1.938 | 620 |
| NHA | 0.015 | 0.008 | 1.875 | 150 |

Aliquots (2ul) from mRNA samples from each of the cell lines were electrophoresed on a 2% agarose gel. Visualisation of the agarose gels typically showed that intact mRNA was obtained from each of the cell lines. Typically, no running streaks were observed on the gels, indicating that there was little or no degradation of the extracted mRNA. The purity of the mRNA (as assessed by the A₂₆₀ / A₂₈₀ ratio) is an important factor to be considered in these experiments because the contaminating presence of cellular proteins can reduce the efficiency of the first strand cDNA synthesis reaction. The A₂₆₀ / A₂₈₀ ratios of all the mRNA samples used in this study were between 1.8 and

2.0, to ensure that the mRNA samples were of an appropriate quality for use in PCR experimentation.

Table 3.3 An example of the obtained spectrophotometric readings and the subsequent concentrations of the mRNA isolated from rat brain tissue at 0, 3, 6, 12 and 24 hour post mortem interval used in this study.

| Post-mortem Interval | A₂₆₀ reading | A₂₈₀ reading | A₂₆₀ / A₂₈₀ ratio | Concentration (ng/μl) |
|-----------------------------|--------------------------------|--------------------------------|--|------------------------------|
| 0 hr | 2.249 | 1.434 | 1.57 | 89.9 |
| 3 hr | 1.563 | 0.872 | 1.79 | 62.5 |
| 6 hr | 2.683 | 1.594 | 1.68 | 107.3 |
| 12 hr | 1.086 | 0.662 | 1.64 | 43.4 |
| 24 hr | 0.533 | 0.312 | 1.68 | 21.3 |

The isolated mRNA samples from each of the brain tissue samples were run on a 2% agarose gel. Results indicated that intact mRNA was obtained from each of the brain tissue samples. The A₂₆₀ / A₂₈₀ ratios of all the mRNA used in this study were between 1.57 and 1.79. Although this was lower than the cell lines, the results of downstream assays were not affected.

3.10 Optimization of Primer concentrations

The efficiencies of sets of primers in PCR are dependent on their concentrations in each PCR reaction mixture (Ponchel *et al*, 2003). Primer concentrations that give the lowest threshold cycle (Ct) value should be selected since lower Ct values correspond to more efficient production of PCR products (Fraga *et al*, 2008).

Primer concentration in an amplification reaction should be between 0.1 and 0.5 μ M. Primer concentrations which are too high can result in mispriming, whereby subsequent extension of misprimed molecules results in non-specific PCR products, or may lead to the production of non-specific products such as primer dimers (Fraga *et al*, 2008). The primer concentrations used in this study were based on the manufacturer's protocol and no additional optimization was required.

Cycling parameters were based on the manufacturer's guidelines and optimized empirically. No additional optimization was required.

Magnesium chloride is generally used in all RT-PCR reactions, with a concentration ranging between 1.5 and 3.0 mM. Although the kits used in this study contained magnesium chloride further optimization was performed using different combinations of template and primers, however results indicated no difference, therefore no further optimization was required.

3.11 Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Real time PCR is a highly sensitive, rapid and accurate method which can be used to monitor small changes in gene expression. Quantitative measurements are taken during the exponential phase when the fluorescence produced is proportional to the accumulation of PCR product. Absolute quantification of a target amplicon may be expressed as a copy number. A positive reaction in real time PCR is detected through the accumulation of a fluorescent signal, referred to as the cycle threshold (Ct), which reflects the number of cycles required for the fluorescent signal to cross a cycle threshold, and hence exceeds the background level. Fluorescence values are recorded during every cycle and represent the amount of amplified product. The more template that is present at the start of the reaction, the fewer number of cycles it will take to reach a point at which a fluorescent signal will be first recorded.

3.11.1 Detection Chemistry used in RT-PCR

The most commonly used methodologies for the detection of amplicons by RT-PCR involves fluorescent dyes. One of the most frequently used dyes, which was employed in this study, is SYBR Green I (Roche). SYBR Green I, when free in solution, displays relatively low fluorescence, but when bound to ds-DNA, its fluorescence increases by 1000 fold. The more ds-DNA that is present, the more binding sites there are for the dye, and hence fluorescence increases in proportion to ds-DNA concentration. As the target sequence is amplified, the increase in concentration of the ds-DNA can be directly measured by the corresponding increase in fluorescence signal.

Measurement of fluorescence at the end of the elongation step of every PCR cycle is performed to monitor the increasing amount of amplified DNA. Following the amplification reaction, PCR products are denatured and SYBR Green fluorescence decreases. If the PCR products consist of molecules of homogeneous length, then only a single thermal transition will be detected. Correlation of fluorescence with the temperature curve (often referred to as a dissociation curve) can be used to differentiate between specific and non-specific amplicons, based on the melting temperature of the reaction end products. Together with a melting curve analysis performed under the same PCR conditions, the SYBR Green I format provides an excellent tool for specific product identification and quantification.

3.11.2 Melting Curve Analysis

Melting curve analysis is performed after the amplification stage, when it is used to assess the dissociation-characteristics of double-stranded DNA during heating. For SYBR Green based detection of amplicons, it is important to run a dissociation curve following the real time PCR, because SYBR Green will detect any double stranded DNA, including primer-dimers, contaminating DNA, and PCR products arising from misannealed primers. By viewing a dissociation curve, as shown in Figure 3.2, it is possible to gain confidence that only the desired amplicon is being detected. Melting curve analysis can be used to identify different reaction products, including nonspecific products and primer-dimers. This is valuable because the presence of secondary nonspecific products and primer-dimers can severely reduce the amplification efficiency and accuracy of the data obtained from the experiment. Primer-dimers can also limit the dynamic range of the desired standard curve through competition for reaction components during amplification. After completion of the amplification reaction, a melting curve is generated by increasing the temperature in small increments and

monitoring the fluorescent signal at each step. As the dsDNA in the reaction denatures, the fluorescence decreases rapidly and significantly. A plot of the negative first derivative of the change in fluorescence ($-dF/dT$, the rate of change of fluorescence) vs. temperature has distinct peaks that correspond to the melting temperature (T_m) of each product.

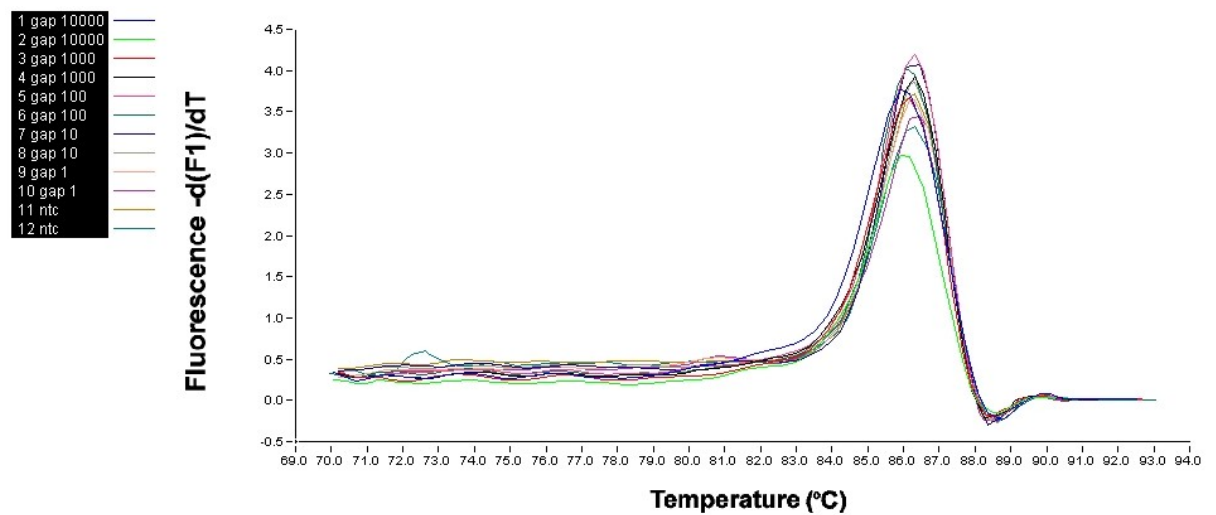


Figure 3.2 An example of a melting (dissociation) curve produced for rat GAPDH. The single peak indicates the presence of one PCR products, no presence of primer-dimers.

3.12. Standard Curve for house keeping gene *GAPDH*

A standard curve is a useful tool for quantifying mRNA and DNA of unknown amounts, and this process is referred to as absolute quantification. Absolute quantification is used to determine how much (number of copies, ng, etc.) of a target gene is present in a particular sample without reference to other samples. In absolute quantification, the quantity of the unknown sample is interpolated from a range of standards of known quantity. To construct a standard curve, a template with known concentration is required. This template is diluted to create a range of standard concentrations.

Absolute quantification is relatively simple and the mathematical calculations are easy to perform. It involves comparing the C_T values of test samples to those of standards of known quantity plotted on a standard curve. Usually, the quantity is normalized to a unit amount of sample, such as the number of cells in the original sample material, volume, or the total amount of nucleic acid. The standard curve constructed from the diluted standard template can then be used to determine the target quantity in the unknown sample by interpolation, in a similar way to that by which molecular size standards are used to determine the molecular size of an unknown DNA band on an agarose gel.

To establish a standard curve, a sample of human and rat genomic DNA was extracted from brain tissue. The size of the human genome was determined as 3,400,000,000 bp and the size of the rat genome was determined as 2,900,000,000 bp from the Database of Genome Sizes (DOGS): <http://www.cbs.dtu.dk/databases/DOGS/index.html>.

For example using the calculation from Applied Biosystems protocol for mass of the rat haploid genome;

(http://www6.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf)

$$M = n \times 1.096 \times e^{-21}$$

Where:

M = Mass of the haploid genome

N = Genome size (bp)

$$e^{-21} = 10^{-21}$$

$$M = 2.9e^9 \times 1.096 \times e^{-21}$$

$$M = 3.1784e^{-12} \text{ g}$$

Convert from g (grams) to pg (picograms)

$$3.1784e^{-12} \text{ g} \times 1e^{12} \text{ pg}$$

$$M = 3.1784 \text{ pg}$$

The mass of the haploid genome was determined as 3.1784 pg. The target gene *GAPDH* exists as a single copy gene per haploid genome and thus two copies per cell (Ponchel *et al*, 2003), and therefore it was calculated that 3.1784 pg of rat genomic DNA contained 1 copy of *GAPDH*. The A_{260} reading was 1.026, and thus the quantity of DNA per μl of original sample was 51,300 pg/ μl . From this, it was calculated that 19.49 μl contained 1 million copy numbers of the *GAPDH* gene sequence.

Table 3.4 Table showing final concentration of genomic DNA required for serial dilution.

| Copy Numbers | Mass of Haploid Genome | Mass of gDNA Needed (pg) | PCR Reaction Volume (μl) | Final Conc. Of gDNA (pg/μl) |
|--------------|------------------------|--------------------------|--------------------------|-----------------------------|
| 10,000 | x 3.1784 | 31784 | / 2 | 15892 |
| 1,000 | | 3178.4 | | 1589.2 |
| 100 | | 317.84 | | 158.92 |
| 10 | | 31.784 | | 15.892 |
| 1 | | 3.1784 | | 1.5892 |

Using the data from Table 3.4, a serial dilution was carried out to give samples containing 1, 10, 100, 1000 and 10,000 copy numbers. The serial dilution was prepared using the formula:

$$C1 \times V1 = C2 \times V2$$

Where:

C1 = Initial Concentration of gDNA (pg/μl)

V1 = Volume of gDNA (μl)

V2 = Final Volume (μl)

C2 = Final Conc. of Dilution (pg/μl)

Table 3.5 Table showing calculation for serial dilution of known copy numbers.

| Source of gDNA | Initial Conc. (pg/μl) | Volume of gDNA (μl) | Volume of Diluent (μl) | Final Volume (μl) | Final Conc. of Dilution (pg/μl) | Resulting Copy No. GAPDH / 2 μl |
|----------------|-----------------------|---------------------|------------------------|-------------------|---------------------------------|---------------------------------|
| | C1 | V1 | | V2 | C2 | |
| Stock | 51300 | 31 | 69 | 100 | 15892 | 10,000 |
| Dilution 1 | 15892 | 10 | 90 | 100 | 1589.2 | 1,000 |
| Dilution 2 | 1589.2 | 10 | 90 | 100 | 158.92 | 100 |
| Dilution 3 | 158.92 | 10 | 90 | 100 | 15.892 | 10 |
| Dilution 4 | 15.892 | 10 | 90 | 100 | 1.5892 | 1 |

A standard curve was produced using RT-PCR utilising the conditions as described in section 2.5 and 2.17, using the crossing points (Figures 3.3 and 3.5) generated from five copy number concentrations of genomic DNA, in triplicate : 1, 10, 100, 1,000 and 10,000 copies with known concentrations (Table 3.5 and 3.6). The equation generated for *GAPDH* for *Rattus norvegicus* ($y = -1.192\ln(x) + 30.35$) (Figure 3.4) was rearranged to $(=EXP ((Ct \text{ value } -30.35/-1.192))$ and for *GAPDH Homo sapiens* ($y = -1.312\ln(x) + 32.05$) (Figure 3.6) was rearranged to $(=EXP ((Ct \text{ value } -32.05/-1.312))$. These were then used to determine copy numbers of *HSPA* and *GAPDH* mRNA expression for rat brain tissue, human cell lines and brain tissue throughout this thesis.

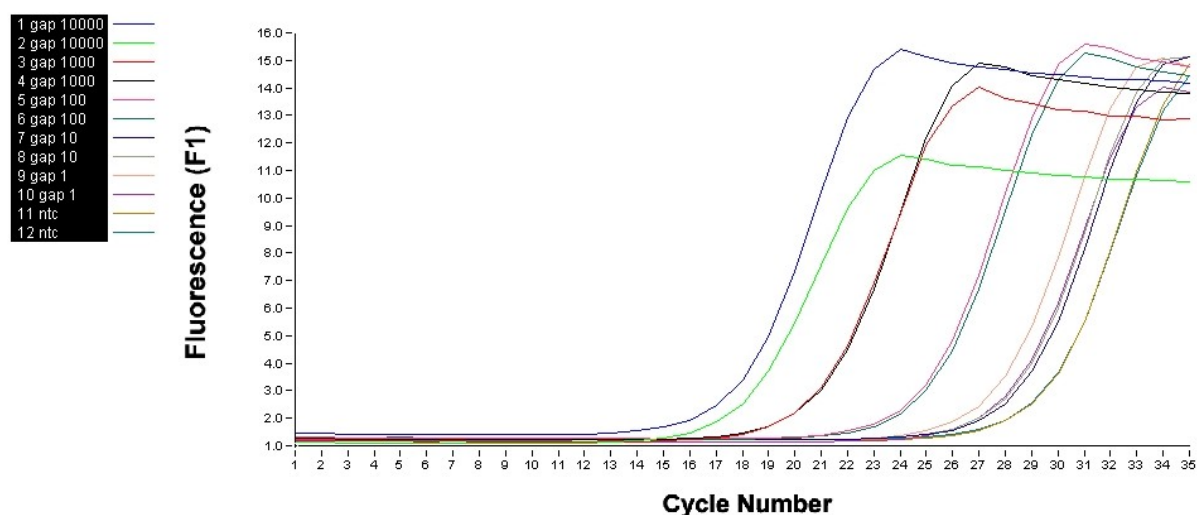


Figure 3.3 Quantification Curve of known concentrations of rat DNA. Standards used to generate the copy numbers of *HSPA*. LightCycler quantification curve generated when known concentration of Genomic rat DNA was amplified, which shows that the higher the concentration the lower the Ct value i.e. earlier detection of fluorescence. The negative control (primer alone, NTC) showed no detection of fluorescence until after 30 Ct (straight line).

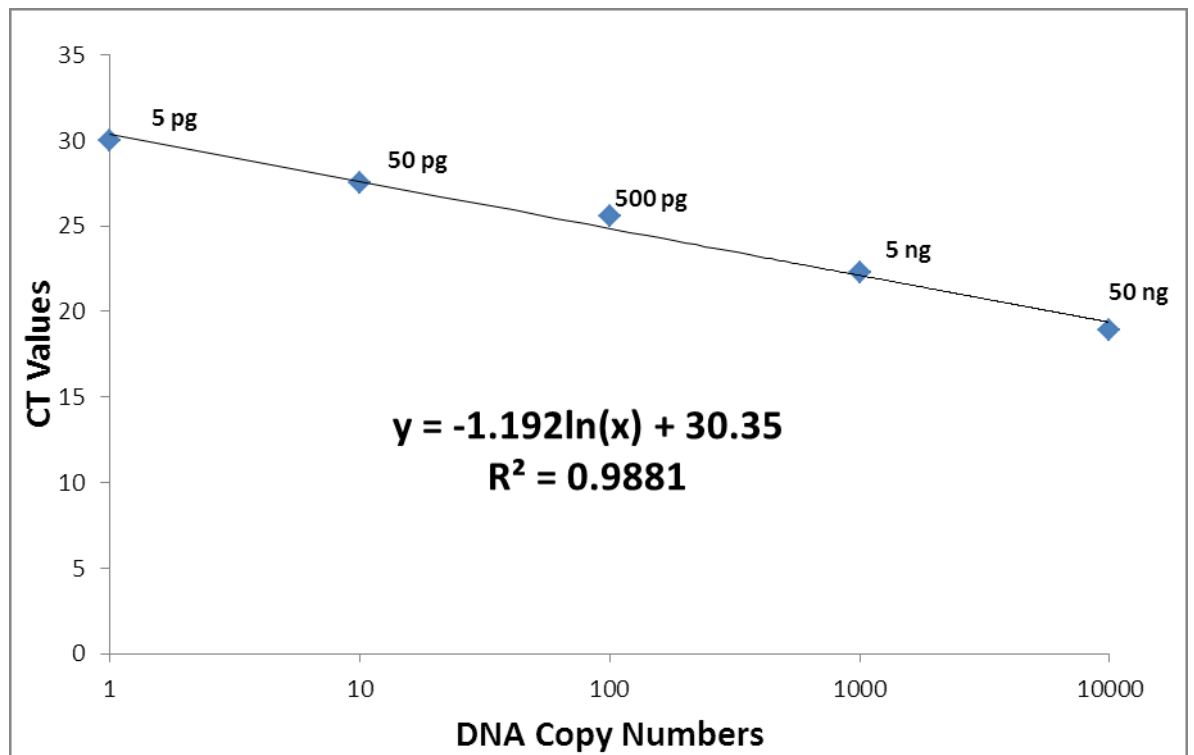


Figure 3.4 Standard curve produced from quantification curve of known concentrations of rat DNA. The standard curve generated from the crossing points showing the relationship between Ct values and copy numbers of the amplified Genomic rat DNA using *GAPDH* reference gene.

Table 3.6 Known concentrations of Genomic human DNA corresponding to the average Ct value and copy number.

| Concentration of Genomic DNA (ng) | Average Ct | Copy number |
|--|-------------------|--------------------|
| 0.005 | 30.15 | 1.7 |
| 0.05 | 29.1 | 17 |
| 0.5 | 26.42 | 170 |
| 5 | 22.6 | 1700 |
| 50 | 18.3 | 17000 |

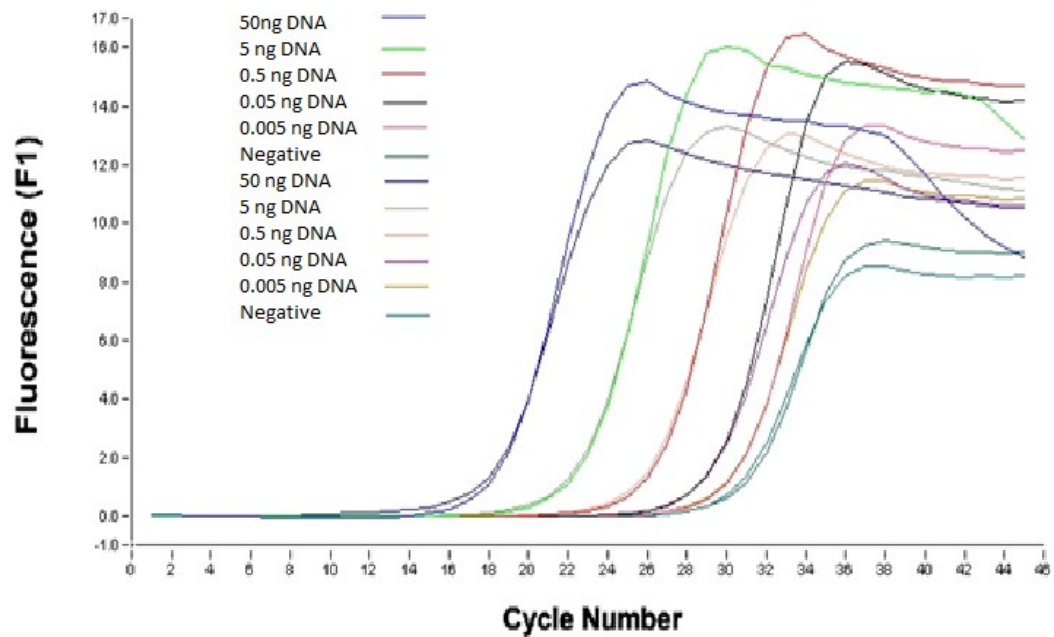


Figure 3.5 Quantification Curve of known concentrations of human DNA. Standards used to generate the copy numbers of *HSPA*. LightCycler quantification curve generated when known concentration of Genomic human DNA was amplified, which shows that the higher the concentration the lower the Ct value i.e. earlier detection of fluorescence. The negative control (primer alone, NTC) showed no detection of fluorescence until after 30 Ct (straight line) (adapted from Mohammed, 2007).

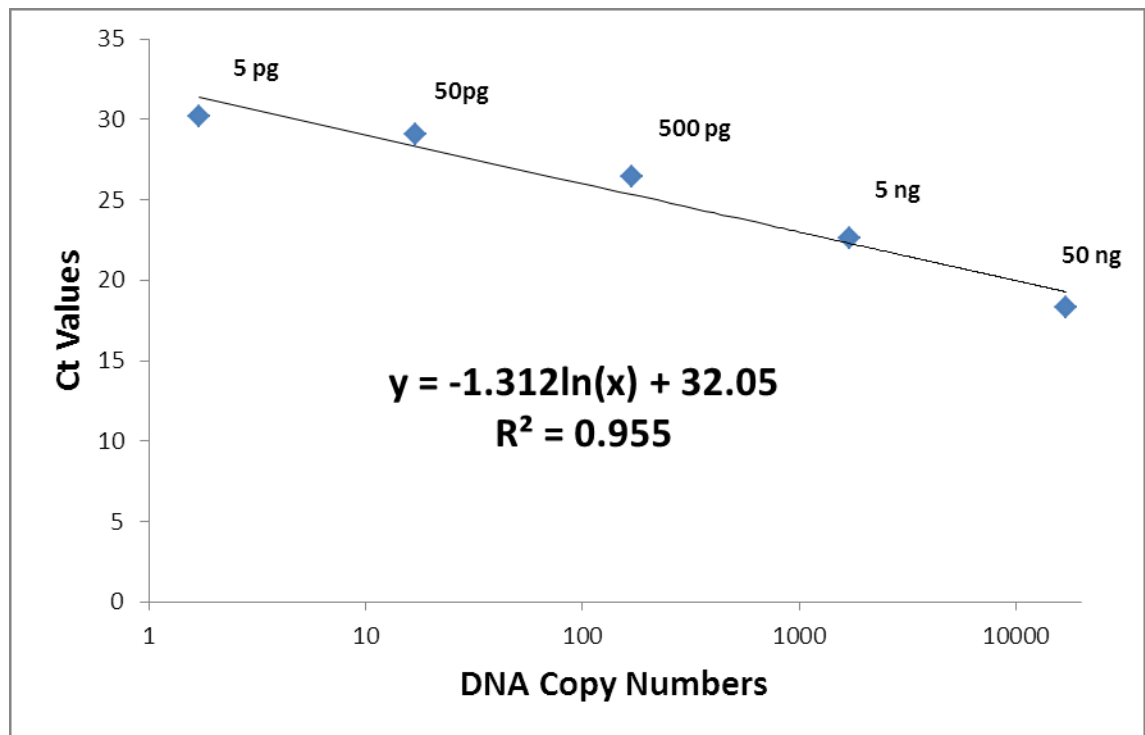


Figure 3.6 Standard curve produced from quantification curve of known concentrations of human DNA. The standard curve generated from the crossing points showing the relationship between Ct values and copy numbers of the amplified Genomic human DNA using *GAPDH* reference gene (adapted from Mohammed, 2007).

3.13 Reference Genes

Control genes, which are often referred to as housekeeping genes, are frequently used to normalise mRNA levels between different tissue types, such as those obtained from normal and diseased cells. The levels of expression of these genes may vary among tissues or cells and may change under certain circumstances (Barber *et al*, 2005; Silver *et al*, 2006). Quantitative studies are commonly used in biomedical research to compare RNA populations under different experimental or clinical conditions. In order to control experimental variations in the amount of RNA used in qRT-PCR, reference (housekeeping) genes are used for the normalization of target gene expression data, such as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), β -actins, and 16S rRNA (Barber *et al*, 2005; Silver *et al*, 2006; Thellin *et al*, 1999). It is assumed that the expression of these genes will remain constant in the cells or tissues under investigation. Although some exceptions to this assumption have been well documented, housekeeping genes are of general value in fully characterized systems. The variability in the expression of the most commonly used housekeeping genes has shown that there is no –one-size-fits-all gene that can be used for normalization of gene expression data (Barber *et al*, 2005; Silver *et al*, 2006). A study undertaken by Barber (2005) has shown that, by providing copy numbers of *GADPH* mRNA expression, the data can be used as a factor for the normalization of gene expression between tissues types, where *GAPDH* is used as the housekeeping control gene. Results indicated differences in *GAPDH* mRNA expression in different tissue types however, the data provided no evidence that there was any effect by age or gender and in most tissues the influence of delay in processing surgical and post-mortem tissues was negligible (Barber *et al*, 2005). Due to the findings within this study, and given that *GAPDH* is one of the most commonly used housekeeping genes used in comparisons of gene expression data, *GAPDH* was chosen as the preferred housekeeping gene to be used throughout this study.

3.14 Real time PCR assay

The Roche LightCycler 2.0 instrument with the LightCycler[®] FastStart^{PLUS} DNA Master SYBR Green I kit was used for quantitative analysis of *HSPA* and *GAPDH* mRNA by real-time PCR. Initial assays were carried out according to the manufacturer's conditions utilising the following optimum parameters: the primer concentration was 1 µM, the annealing temperature for the amplification of *Homo sapien HSPA* and *GAPDH* was 57°C and 56°C respectively and *Rattus norvegicus HSPA* and *GAPDH* 56°C and 57°C was respectively. Confirmation of PCR products was performed by incorporating a melting curve analysis step to ensure that only the target genes were amplified and detected.

Chapter 4

***HSPA* gene expression in glioma cells and brain tissue**

4.1 Gene Expression

HSPA expression is up-regulated in cancer cells (Graner *et al*, 2007; Jolly and Morimoto, 2000), and thus the main research aim of work presented in this chapter was to measure and compare *HSPA* expression in normal and glioma cell lines and normal and cancerous brain tissue. A significant difference between these tissues might allow assessment of its possible use as a prognostic marker for grade related brain tumours.

Tumour and normal brain tissue samples were obtained from patients admitted to the Royal Preston Hospital, UK. Informed consent and ethical approval was obtained prior to this investigation. Tissues were dissected and immediately frozen to -80°C and stored for analysis. In total 18 tissue samples were used in this study (Table 4.5). Human brain cell lines GOS-3 (grade II/III oligodendroglioma) from DMSZ (Germany), U87-MG (grade IV glioblastoma) from ECCAC (UK) and NHA (normal human astrocytes) from Lonza (UK) were also used in this study. GOS-3 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% FBS and with 4 mM L-glutamide, while U87-MG cells were cultured in Eagle's minimum essential medium (EMEM) (Sigma) supplemented with 2 mM L-glutamide, 10% FBS and 1% (v/v) nonessential amino acids (Sigma). The NHA cells were cultured in astrocyte medium (AM) supplemented with 15 ml of FBS, 0.5 ml Ascorbic Acid, 0.5 ml rhEGF (astrocyte growth supplement) 0.5 ml GA100 1.25 ml Insulin and 5 ml L-glutamide.

mRNA was isolated from all four cell lines, 15 human glioma brain tissues and three normal (control) brain tissues using mRNA Isolation Kit (Roche, UK) following the manufacturer's protocol. The concentration and purity of mRNA was determined by ultraviolet spectrophotometry. Isolated mRNA (100 ng) was transcribed to cDNA using

1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche, UK) following the manufacturer's protocol, which was then used as a template for qRT-PCR. Quantitative real-time PCR was used to evaluate the expression of *HSPA*, and *GAPDH* as a control using FastStart DNA Master^{PLUS} SYBR Green 1 (Roche, UK). Primers used for *HSPA* were 5' CGACCTGAACAAGAGCATCA 3' (sense) and 5' AAGATCTGCGTCTGCTTGGT 3' (antisense). For *GAPDH*, primers were 5' GAGTCAAGCGATTTGGTCGT 3' (sense) and 5' TTGATTTTGGAGGGATCTCG 3' (antisense). All primers were designed using Primer3 software and manufactured by TIB MOLBIOL. After an initial denaturation at 95°C for 10 min, the samples were subjected to 35 cycles of RT-PCR 95°C for 10 s, annealing temperature 57°C (*HSPA*) and 56°C (*GAPDH*) for 15 s, and 72°C for 15 s (Patel *et al.*, 2008). At the end of each cycle, the fluorescence emitted was measured in a single step in channel F1 (gain1). After the 35th cycle, the specimens were heated to 95°C and rapidly cooled to 65°C for 15 s. All heating and cooling steps were performed with a slope of 20°C / s. The temperature was subsequently raised to 95°C with a slope of 0.1°C / s and fluorescence was measured continuously (channel F1, gain1) to obtain data for the melting curve analysis. All PCR reactions were performed in triplicate and a negative control included, which contained primers with no DNA. All PCR products were analysed using gel electrophoresis stained and visualised using a gel analyser (SynGene, UK).

Expression of *HSPA* was compared between the three glioma cell lines (1321N1, GOS-3 and U87-MG) and in a normal (control) human astrocyte cell line (NHA), and also in 15 glioma and 3 normal brain tissue samples. *GAPDH* was used as an internal standard throughout all RT-PCR experiments (Barber *et al.*, 2005).

4.2 Constitutive Expression

4.2.1 Glioma Cell lines

Copy numbers of *HSPA* and *GAPDH* transcripts were determined using RT-PCR in the three glioma cell lines (1321N1, GOS-3 and U87-MG), and in the normal human astrocyte cell line (NHA). The primers and optimal temperatures used for the amplification of both genes are documented in Table 2.10 in section 2.5. All PCR experiments were carried out in triplicate for consistency and repeatability. For each gene analysed, a quantification graph was produced to confirm gene amplification. The resulting amplicons for *HSPA* and *GAPDH* were also visualized using agarose gel electrophoresis, each being represented by bands of 213 and 238 bp, respectively. The three glioma cell lines transcribed *HSPA* at higher levels than the normal astrocyte cell line NHA (Figure 4.1). *GAPDH* transcript levels were consistent in all glioma and normal cell lines, confirming comparability of *HSPA* results (Figure 4.1). The mRNA copy number for both genes was calculated for each cell line to monitor the gene expression level. For *HSPA*, mRNA copy numbers per 100 ng of extracted mRNA confirmed that 1321N1, produced approximately 7,500 copies, GOS-3 contained approximately 8,900 copies and U87-MG contained approximately 8,200 copies of this transcript. Glioma cell lines expressed *HSPA* at a higher level than normal human astrocyte NHA, with approximately 1,400 copies, indicating that *HSPA* is expressed at lower levels in normal cells but is highly expressed in cancer cells (Table 4). For *GAPDH*, mRNA copy numbers per 100 ng of extracted mRNA were relatively consistent, NHA produced approximately 146,500 copies, 1321N1 contained approximately 144,500 copies, GOS-3 contained approximately 147,500 copies and U87-MG contained approximately 147,500 copies, again confirming comparability of *HSPA* results (Table 4.1).

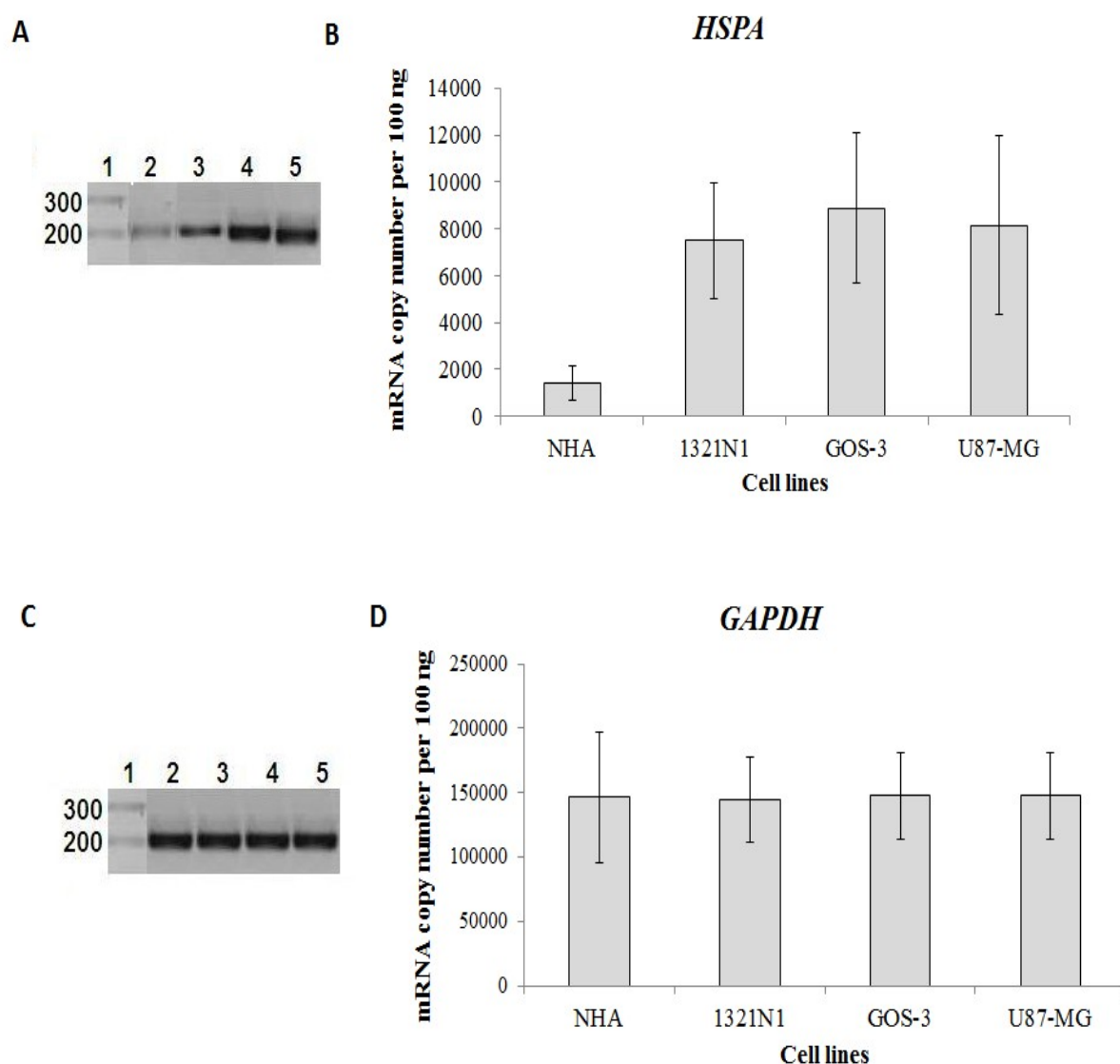


Figure 4.1 *HSPA* and *GAPDH* transcript levels in NHA, 1321N1, GOS-3 and U87-MG cell lines. A (*HSPA*) and C (*GAPDH*), show agarose gel electrophoresis of samples. Lane 1 represents the 100 bp molecular ladder, lanes 2 – 5 represents amplicons from NHA, 1321N1, GOS-3 and U87-MG respectively. B and D histograms represent mRNA copy numbers per 100 ng of extracted mRNA of *HSPA* (B) and *GAPDH* (D) in the same cell lines. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars.

4.2.2 Statistical Analysis

A one-way ANOVA test between groups of analysis of variance was conducted to analyse results of the previous experiments of *HSPA* and *GAPDH* gene expression in normal (control) and glioma cell lines in three independent experiments. For this statistical analysis, the cell lines comprised of four groups: group 1 NHA, group 2 1321N1, group 3 GOS-3 and group 4 U87-MG. The means and standard deviations are presented in Table 4.1 (*HSPA*) and Table 4.3 (*GAPDH*).

Table 4.1 Descriptive statistics showing the mean expression levels of *HSPA* for each cell line.

| Cell Line | N | Mean <i>HSPA</i> transcript copy number | Std Deviation |
|-----------|---|---|---------------|
| NHA | 3 | 1419.63 | 708.76 |
| 1321N1 | 3 | 7491.67 | 2495.20 |
| GOS-3 | 3 | 8879.29 | 3210.83 |
| U87-MG | 3 | 8158.12 | 3816.44 |

There was a significant difference in *HSPA* transcript copy number between the four cell line groups [$F(3,8) = 5.157$, $P = 0.028$]. The effect size, calculated using eta squared indicated a large effect of 0.659.

Table 4.2. Statistical data from Tukey's HSD test showing significance of *HSPA* gene expression between cell lines. Any significant differences are highlighted in bold.

| (I) Cell | J (Cell) | Mean Difference between copy number (I – J) | Std. Error | Sig. |
|------------|--------------|---|----------------|--------------|
| NHA | 1321N1 | 6072.04 | 2133.21 | 0.083 |
| NHA | GOS-3 | 7459.67 | 2133.21 | 0.033 |
| NHA | U87-MG | 6738.49 | 2133.21 | 0.053 |
| 1321N1 | GOS-3 | 1387.62 | 2133.21 | 0.912 |
| 1321N1 | U87-MG | 666.45 | 2133.21 | 0.989 |
| GOS-3 | U87-MG | 721.17 | 2133.21 | 0.986 |

Post-hoc analyses using Tukey's HSD test indicated significantly lower *HSPA* gene expression in group 1, NHA than in group 3, GOS-3 (MD = 7459.67, P = 0.033). All other differences between groups failed to reach significance (Table 4.2). Significance \leq 0.05.

Table 4.3 Descriptive statistics showing the mean transcript levels of *GAPDH* in each cell line.

| Cell Line | N | Mean <i>GAPDH</i> transcript copy number | Std Deviation |
|------------------|----------|---|----------------------|
| NHA | 3 | 146468.91 | 50836.00 |
| 1321N1 | 3 | 144546.62 | 18955.42 |
| GOS-3 | 3 | 147541.72 | 19549.02 |
| U87-MG | 3 | 147541.72 | 19549.02 |

There was no significant difference in *GAPDH* gene expression between the four cell line groups [$F(3,8) = 0.004$, $P > .05$]. The effect size, calculated using eta squared indicated a small effect of 0.001.

Table 4.4. Statistical data from Tukey's HSD test showing significance of *GAPDH* gene expression between cell lines.

| (I) Cell | J (Cell) | Mean Difference between copy number (I – J) | Std. Error | Sig. |
|-----------------|-----------------|--|-------------------|-------------|
| NHA | 1321N1 | 1922.29 | 31504.52 | 1.000 |
| NHA | GOS-3 | 1072.82 | 31504.52 | 1.000 |
| NHA | U87-MG | 1072.82 | 31504.52 | 1.000 |
| 1321N1 | GOS-3 | 2995.10 | 31504.52 | 1.000 |
| 1321N1 | U87-MG | 2995.10 | 31504.52 | 1.000 |
| GOS-3 | U87-MG | 0.00 | 31504.52 | 1.000 |

Post-hoc analyses using Tukey's HSD test indicated no significant difference in *GAPDH* gene expression between any of the four groups (Table 4.4). Significance ≤ 0.05 .

4.2.3 Brain Tissue Samples

The expression of *HSPA* and *GAPDH* were analysed in eighteen brain tissue specimens, of which fifteen were glioma tissues and three were normal (control) brain tissue samples. RNA populations were again analysed by RT-PCR, with the mRNA copy number for each gene being calculated for each sample, sections 2.7 and 3.11.

Table 4.5 Tissues used in this study.

| | Diagnosis | Grade | Age | Gender | Survival from diagnosis to death |
|-----|--|-------|-----|--------|-------------------------------------|
| G1 | glioblastoma | IV | 16 | F | (Alive) 13+ months |
| G2 | glioblastoma | IV | 23 | F | 10 months |
| G3 | glioblastoma | IV | 38 | M | 8 months |
| G4 | glioblastoma | IV | 38 | M | 10 months |
| G5 | glioblastoma | IV | 46 | F | 11 months |
| G6 | glioblastoma | IV | 60 | F | 10 months |
| G7 | glioblastoma | IV | 62 | M | 11 months |
| G8 | glioblastoma | IV | 63 | M | 10 months |
| G9 | glioblastoma | IV | 64 | M | 11 months |
| G10 | glioblastoma | IV | 67 | F | 12 months |
| G11 | glioblastoma | IV | 68 | M | 8 months |
| G12 | glioblastoma | IV | 28 | F | 10 months |
| G13 | recurrent anaplastic ependymoma | III | 34 | F | 8 months |
| G14 | anaplastic oligodendroglioma III | III | 56 | M | 11 months |
| G15 | anaplastic oligoastrocytoma III | III | 62 | F | 11 months |
| G16 | normal | | 39 | F | Alive |
| G17 | normal | | 38 | F | Alive |
| G18 | normal | | 66 | M | Alive |

The results showed that tissue samples from 8 of the 12 glioblastoma patients showed high levels, (> 2000 copies), of *HSPA* transcripts, with approximate copy numbers per 100 ng of extracted mRNA ranging between 2,100 and 3,300, while the remaining tissue samples from the 4 glioblastoma patients showed lower levels, (< 900 copy numbers), of *HSPA* transcripts, with approximate copy numbers per 100 ng of extracted mRNA ranging between 300 and 870 (Figure 4.2 and Table 4.6). The tissue sample from the ependymoma patient showed high levels of *HSPA* transcripts, approximate copy number per sample, G13 2,000, whilst the tissue samples from the oligodendroglioma III patient, and from the oligoastrocytoma III patient, had low but detectable *HSPA* transcript levels, with approximate copy numbers per 100 ng of extracted mRNA of G14 750 and G15 350 respectively (Figure 4.2 and Table 4.6). The three control samples also showed relatively low or barely detectable levels of *HSPA* transcripts, with approximate copy numbers per 100 ng of extracted mRNA ranging between 100 and 400 (Figure 4.2 and Table 4.6). *HSPA* transcription in glioma samples was independent of age or gender. The mRNA copy numbers of *HSPA* were averaged for the twelve glioblastomas and compared against similar average data from the three low grade gliomas and three control brain tissues samples (Figure 4.4). Significantly higher *HSPA* transcription was observed in the glioblastoma tissues compared to low grade tissue samples, (approximately 1.75-fold higher), and the control tissue samples, (approximately 9-fold higher). Significantly higher *HSPA* transcription was observed in the low grade tissue samples compared to control tissue samples, (approximately 5-fold higher); (Figure 4.4).

As seen in the glioma cell lines, section 4.2.1, more *HSPA* transcripts were observed in the glioma tissues, (approximately 6-fold higher), than in control brain tissue, confirming that *HSPA* expression is consistently up-regulated in some cancer cells. For

GAPDH, mRNA copy numbers per 100 ng of extracted mRNA were relatively consistent in all brain tissue samples, with approximately 143,700 copies per 100 ng of extracted mRNA, giving confidence in the comparability of *HSPA* experiments (Figure 4.3 and Table 4.6).

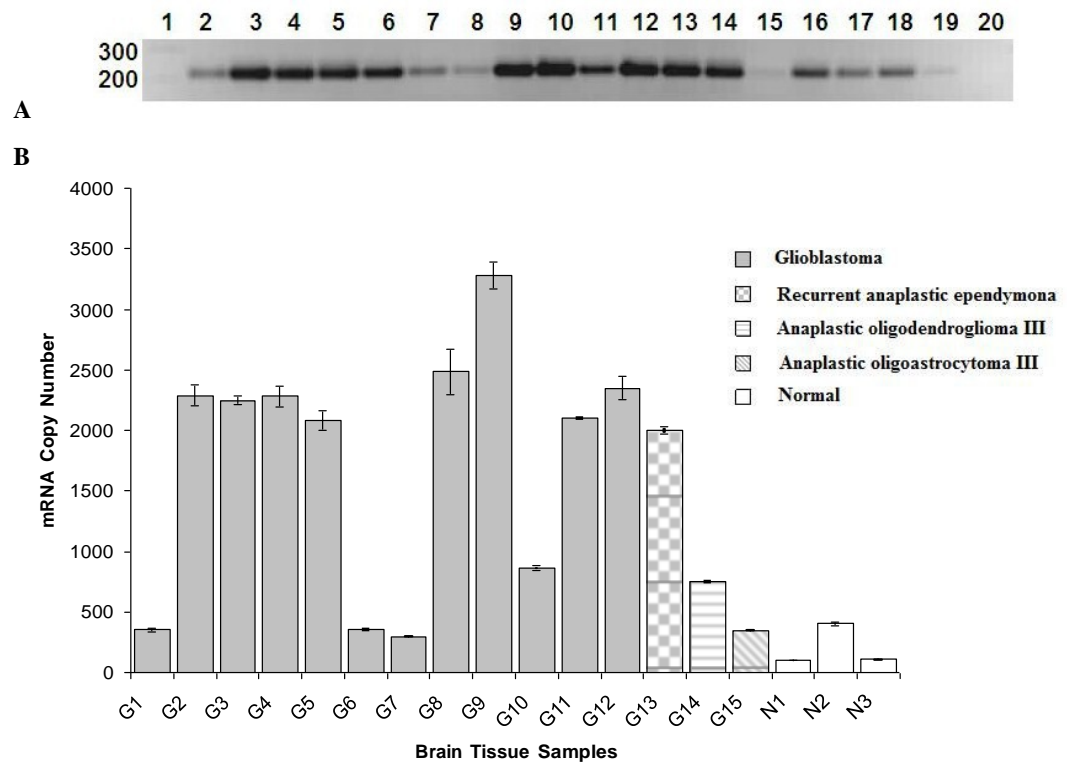


Figure 4.2 *HSPA* mRNA copy number in 18 brain tissue samples. A. Agarose gel electrophoresis: Lane 1 represents the 100 bp molecular ladder; lanes 2 - 13 represent amplicons from glioblastoma tissues; lanes 14 - 16 represent amplicons from ependymoma, oligodendroglioma III and oligoastrocytoma III tissues respectively; lanes 17 – 19 represent amplicons from normal brain tissues and lane 20 is a no template control. B. Histogram representing mRNA copy numbers per 100 ng of extracted mRNA of *HSPA* in tissues. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars. G1 – G12 represents the brain tissue of individual patients with glioblastoma, G13 represents the brain tissue from an individual patient with ependymoma, G14 represents the brain tissue from an individual patient with oligodendroglioma III, G15 represents the brain tissue from an individual patient with oligoastrocytoma III and N1 – N3 represents the normal (control) brain tissue from individual .

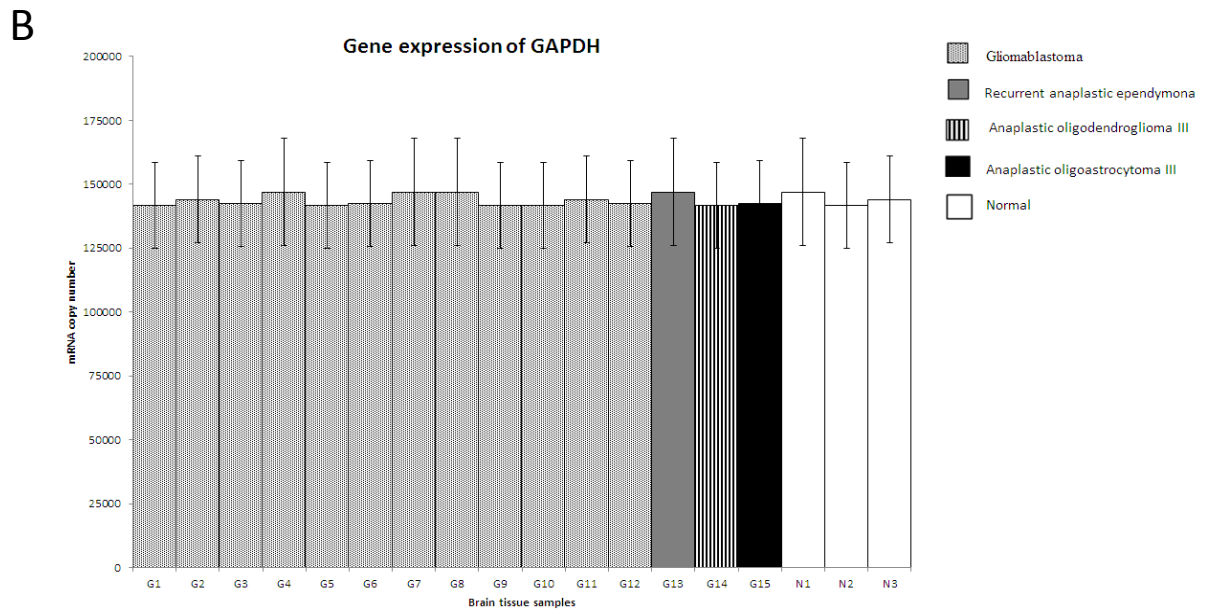
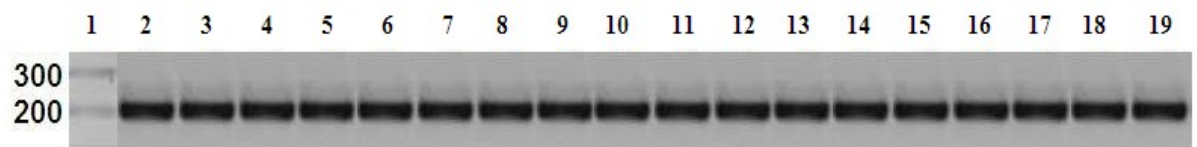


Figure 4.3 *GAPDH* mRNA in 18 brain tissue samples. A. Agarose gel electrophoresis: Lane 1 represents the 100 bp molecular marker; lanes 2 - 13 represent amplicons from glioblastoma tissues; lanes 14 - 16 represent amplicons from ependymoma, oligodendroglioma III and oligoastrocytoma III tissues respectively; lanes 17 – 19 represent amplicons from normal brain tissue. B. Histogram representing mRNA copy numbers per 100 ng of extracted mRNA of *GAPDH* in tissues. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars. G1 – G12 represents the brain tissue of individual patients with glioblastoma, G13 represents the brain tissue from an individual patient with ependymoma, G14 represents the brain tissue from an individual patient with oligodendroglioma III, G15 represents the brain tissue from an individual patient with

oligoastrocytoma III and N1 – N3 represents the normal (control) brain tissue from individual .

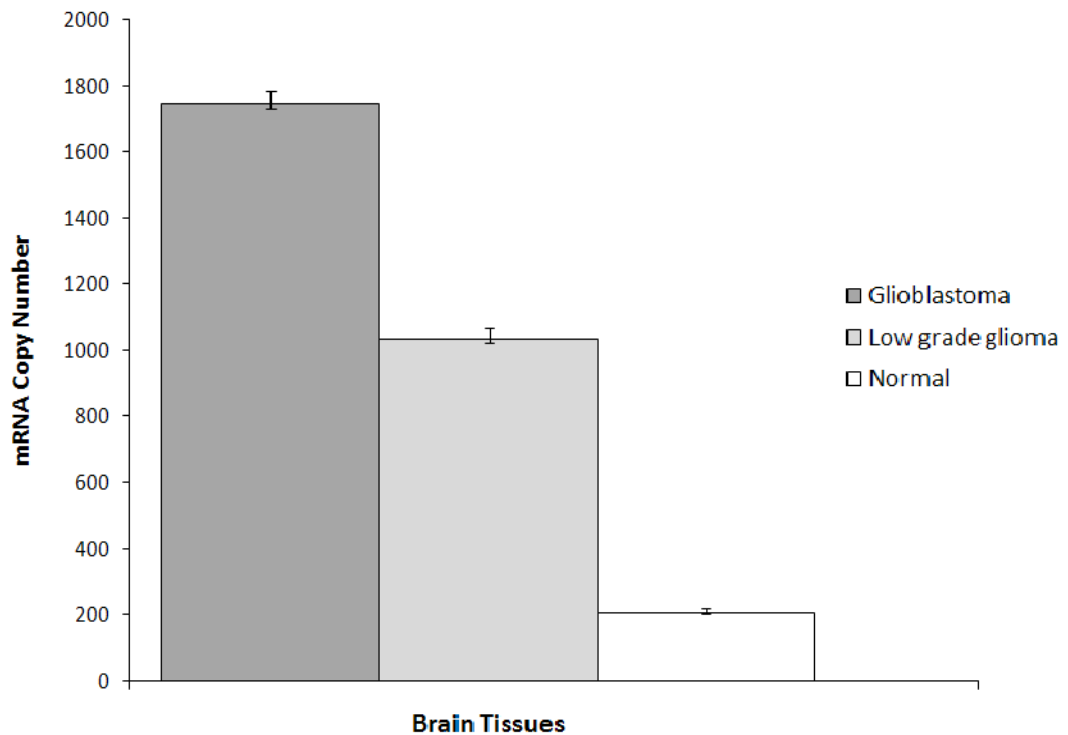


Figure 4.4 Histogram showing the mean *HSPA* mRNA copy numbers in brain tissues grouped by: Glioblastomas (n = 12), low grade gliomas (n = 3) and normal tissues (n = 3). mRNA copy number data from 12 glioblastoma tissues were averaged and compared against the three low grade gliomas and the three normal brain tissues. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars.

As can be seen in Figure 4.4 *HSPA* transcripts are approximately 9-fold higher in the glioblastoma tissues compared to the control tissue samples. *HSPA* transcripts are also approximately 5-fold higher in the low grade tissue samples than in control tissue samples.

4.2.4 Statistical Analysis

A one-way ANOVA test between groups of analysis of variance was conducted to analyse results of the previous experiments of *HSPA* and *GAPDH* gene expression in normal (control) and glioma brain tissue in three independent experiments. For this statistical analysis, the brain tissue samples comprised eighteen groups: groups G1 – G12, glioblastomas; group G13, recurrent anaplastic ependymoma; group G14, anaplastic oligodendroglioma III; G15, anaplastic oligoastrocytoma III and groups N1 – N3 normal brain tissue. The means and standard deviations for *HSPA* and *GAPDH* results are presented in Table 4.6.

There was significant differences in *HSPA* transcript copy numbers between the eighteen brain tissue groups [$F(17,36) = 719.138$, $P < 0.001$]. The effect size, calculated using eta squared indicated a large effect of 0.997.

Data obtained from post-hoc analyses using Tukey's HSD test indicated significantly lower *HSPA* transcript copy numbers in G1 than in: G2 (MD = 1935.13, $P < .05$); G3 (MD = 1893.88, $P < .05$); G4 (MD = 1929.31, $P < .05$); G5 (MD = 1729.89, $P < .05$); G8 (MD = 2132.44, $P < .05$); G9 (MD = 2928.44, $P < .05$); G10 (MD = 514.00, $P < .05$); G11 (MD = 1750.16, $P < .05$); G12 (MD = 1994.21, $P < .05$); G13 (MD = 1651.22, $P < .05$); G14 (MD = 397.09, $P < .05$) and N1 (MD = 247.85, $P < .05$) (Table 4.7).

There was significantly lower *HSPA* transcript copy numbers in G2 than in: G6 (MD = 1935.24, $P < .05$); G7 (MD = 1991.97, $P < .05$); G9 (MD = 993.31, $P < .05$); G10 (MD = 1421.12, $P < .05$); G13 (MD = 283.90, $P < .05$), G14 (MD = 1538.03), G15 (MD = 1937.91, $P < .05$); N1 (MD = 2182.97, $P < .05$), N2 (MD = 1883.25, $P < .05$) and N3 (MD = 2180.54, $P < .05$) (Table 4.7).

There was significantly lower *HSPA* transcript copy numbers in G3 than in: G6 (MD = 1893.99, $P < .05$); G7 (MD = 1950.73, $P < .05$); G8 (MD = 238.58, $P < .05$); G9 (MD = 1034.55, $P < .05$); G10 (MD = 1379.88, $P < .05$); G13 (MD = 242.66, $P < .05$); G14 (MD = 1496.79, $P < .05$); G15 (MD = 1896.67, $P < .05$); N1 (MD = 2141.73, $P < .05$); N2 (MD = 1842.01, $P < .05$) and N3 (MD = 2139.30, $P < .05$) (Table 4.7).

There was significantly lower *HSPA* transcript copy numbers in G4 than in: G6 (MD = 1929.42, $P < .05$); G7 (MD = 1986.15, $P < .05$); G9 (MD = 999.13, $P < .05$); G10 (MD = 1415.31, $P < .05$); G13 (MD = 278.09, $P < .05$); G14 (MD = 1532.22, $P < .05$); G15 (MD = 1932.10, $P < .05$); N1 (MD = 2177.16, $P < .05$); N2 (MD = 1877.44, $P < .05$) and N3 (MD = 2174.72, $P < .05$) (Table 4.7).

There was significantly lower *HSPA* transcript copy numbers in G5 than in: G6 (MD = 1730.00, $P < .05$); G7 (MD = 1786.74, $P < .05$); G8 (MD = 402.55, $P < .05$); G9 (MD = 1198.54, $P < .05$); G10 (MD = 1215.89, $P < .05$); G12 (MD = 264.32, $P < .05$); G14 (MD = 1332.80, $P < .05$); G15 (MD = 1732.68, $P < .05$); N1 (MD = 1977.74, $P < .05$), N2 (MD = 1678.02, $P < .05$) and N3 (MD = 1975.31, $P < .05$) (Table 4.7).

There was significantly lower *HSPA* transcript copy numbers in G6 than in: G8 (MD = 2132.55, $P < .05$); G9 (MD = 2928.55, $P < .05$); G10 (MD = 514.11, $P < .05$); G11 (MD = 1750.27, $P < .05$); G12 (MD = 1994.32, $P < .05$); G13 (MD = 1651.33, $P < .05$); G14 (MD = 397.20, $P < .05$); N1 (MD = 247.74, $P < .05$) and N3 (MD = 245.31, $P < .05$) (Table 4.7).

There was significantly lower *HSPA* transcript copy numbers in G7 than in: G8 (MD = 2189.28, $P < .05$); G9 (MD = 2985.28, $P < .05$); G10 (MD = 570.85, $P < .05$); G11 (MD = 1807.00, $P < .05$); G12 (MD = 2051.06, $P < .05$); G13 (MD = 1708.07, $P < .05$) and G14 (MD = 453.94, $P < .05$) (Table 4.7).

There was significantly lower *HSPA* transcript copy numbers in G8 than in: G9 (MD = 796.00, $P < .05$); G10 (MD = 1618.44, $P < .05$); G11 (MD = 382.28, $P < .05$); G13 (MD = 481.22, $P < .05$); G14 (MD = 1735.35, $P < .05$); G15 (MD = 2135.23, $P < .05$); N1 (MD = 2380.29, $P < .05$); N2 (MD = 2080.57, $P < .05$) and N3 (MD = 2377.85, $P < .05$) (Table 4.7).

There was significantly lower *HSPA* transcript copy numbers in G9 than in: G10 (MD = 2414.43, $P < .05$); G11 (MD = 1178.28, $P < .05$); G12 (MD = 934.26, $P < .05$); G13 (MD = 1277.21, $P < .05$); G14 (MD = 2531.24, $P < .05$); G15 (MD = 2931.22, $P < .05$); N1 (MD = 3176.28, $P < .05$); N2 (MD = 2876.56, $P < .05$) and N3 (MD = 3173.85, $P < .05$) (Table 4.7).

There was significantly lower *HSPA* transcript copy numbers in G10 than in: G11 (MD = 1236.16, $P < .05$); G12 (MD = 1480.21, $P < .05$); G13 (MD = 1137.22, $P < .05$); G15 (MD = 526.79, $P < .05$); N1 (MD = 761.85, $P < .05$); N2 (MD = 462.13, $P < .05$) and N3 (MD = 759.41, $P < .05$) (Table 4.7).

There was significantly lower *HSPA* transcript copy numbers in G11 than in: G12 (MD = 244.05, $P < .05$); G14 (MD = 1353.07, $P < .05$); G15 (MD = 1752.95, $P < .05$); N1 (MD = 1998.01, $P < .05$); N2 (MD = 1698.29, $P < .05$) and N3 (MD = 1995.57, $P < .05$) (Table 4.7).

There was significantly lower *HSPA* transcript copy numbers in G12 than in: G13 (MD = 342.99, $P < .05$); G14 (MD = 1597.12, $P < .05$); G15 (MD = 1997.00, $P < .05$); N1 (MD = 2242.06, $P < .05$); N2 (MD = 1942.34, $P < .05$) and N3 (MD = 2239.62, $P < .05$) (Table 4.7).

There was significantly lower *HSPA* transcript copy numbers in G13 than in: G14 (MD = 1254.13, $P < .05$); G15 (MD = 1654.01, $P < .05$); N1 (MD = 1899.07, $P < .05$); N2 (MD = 1599.35, $P < .05$) and N3 (MD = 1896.64, $P < .05$) (Table 4.7).

There was significantly lower *HSPA* transcript copy numbers in G14 than in: G15 (MD = 399.88, $P < .05$); N1 (MD = 644.94, $P < .05$); N2 (MD = 345.22, $P < .05$) and N3 (MD = 642.51, $P < .05$) (Table 4.7).

There was significantly lower *HSPA* transcript copy numbers in G15 than in: N1 (MD = 245.06, $P < .05$) and N3 (MD = 242.63, $P < .05$) (Table 4.7).

There was significantly lower *HSPA* transcript copy numbers in N1 than in N2 (MD = 299.72, $P < .05$) (Table 4.7).

There was significantly lower *HSPA* transcript copy numbers in N2 than in N3 (MD = 297.29, $P < .05$) (Table 4.7).

All other difference between groups failed to reach significance (Table 4.7).

There was a significant difference in *GAPDH* transcript copy numbers between the eighteen brain tissue groups [$F(17,36) = 0.43$, $P = >.05$]. The effect size, calculated using eta squared indicated a small effect of 0.02.

Post-hoc analyses using Tukey's HSD test indicated no significant difference ($P > .05$) in *GAPDH* transcript copy numbers between any of the eighteen groups.

Table 4.6 Descriptive statistics showing the mean transcript copy numbers of *HSPA* and *GAPDH* expression in brain tissue sample.

| Tissue sample | N | Mean <i>HSPA</i> gene expression | Std Deviation | Mean <i>GAPDH</i> gene expression | Std Deviation |
|----------------------|----------|---|----------------------|--|----------------------|
| G1 | 3 | 352.86 | 13.44 | 141748.41 | 16746.50 |
| G2 | 3 | 2287.99 | 87.40 | 143942.85 | 16976.61 |
| G3 | 3 | 2246.74 | 34.24 | 142468.77 | 16727.43 |
| G4 | 3 | 2282.17 | 86.93 | 146885.07 | 20948.44 |
| G5 | 3 | 2082.76 | 79.34 | 141748.41 | 16746.50 |
| G6 | 3 | 352.75 | 8.06 | 142468.77 | 16727.43 |
| G7 | 3 | 296.02 | 4.51 | 146885.07 | 20948.44 |
| G8 | 3 | 2485.30 | 189.23 | 146885.07 | 20948.44 |
| G9 | 3 | 3281.30 | 112.51 | 141748.41 | 16746.50 |
| G10 | 3 | 866.86 | 19.81 | 141748.41 | 16746.50 |
| G11 | 3 | 2103.02 | 9.26 | 143942.85 | 16976.61 |
| G12 | 3 | 2347.07 | 98.28 | 142468.77 | 16727.43 |
| G13 | 3 | 2004.08 | 30.54 | 146885.07 | 20948.44 |
| G14 | 3 | 749.95 | 11.43 | 141748.41 | 16746.50 |
| G15 | 3 | 350.07 | 8.00 | 142468.77 | 16727.43 |
| N1 | 3 | 105.01 | .800 | 146885.07 | 20948.44 |
| N2 | 3 | 404.73 | 15.42 | 141748.41 | 16746.50 |
| N3 | 3 | 107.45 | 1.64 | 143942.85 | 16976.61 |

Table 4.7 Statistical data from Tukey’s HSD test showing significance of *HSPA* transcript copy numbers between brain tissue sample groups. Any significant differences ($P \leq 0.05$) between groups are highlighted bold.

| | G1 | G2 | G3 | G4 | G5 | G6 | G7 | G8 | G9 | G10 | G11 | G12 | G13 | G14 | G15 | N1 | N2 | N3 |
|----|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| G1 | | M1935.12 Sig .000 | M1893.88 Sig .000 | M1929.31 Sig .000 | M1729.89 Sig .000 | M.109 Sig 1.0 | M56.84 Sig 1.0 | M2132.44 Sig .000 | M2928.44 Sig .000 | M514.00 Sig .000 | M1750.16 Sig .000 | M1994.21 Sig .000 | M1651.22 Sig .000 | M397.09 Sig .000 | M2.79 Sig 1.0 | M247.85 Sig.008 | M51.87 Sig 1.0 | M245.41 Sig.009 |
| G2 | M1935.12 Sig .000 | | M41.24 Sig 1.0 | M5.82 Sig 1.0 | M205.23 Sig .056 | M1935.24 Sig .000 | M1991.97 Sig .000 | M197.31 Sig .078 | M993.31 Sig .000 | M1421.12 Sig .000 | M184.97 Sig .129 | M59.08 Sig 1.0 | M283.90 Sig .001 | M1538.03 Sig .000 | M1937.91 Sig .000 | M2182.97 Sig .000 | M1883.25 Sig .000 | M2180.54 Sig .000 |
| G3 | M1893.88 Sig .000 | M41.24 Sig 1.0 | | M35.43 Sig 1.0 | M163.99 Sig .271 | M1893.99 Sig .000 | M1950.73 Sig .000 | M238.56 Sig .012 | M1034.55 Sig .000 | M1379.88 Sig .000 | M143.72 Sig .483 | M100.33 Sig .925 | M242.66 Sig .010 | M1496.79 Sig .000 | M1896.67 Sig .000 | M2141.73 Sig .000 | M1842.01 Sig .000 | M2139.30 Sig .000 |
| G4 | M1929.31 Sig .000 | M5.82 Sig 1.0 | M35.43 Sig 1.0 | | M199.42 Sig .072 | M1929.42 Sig .000 | M1986.15 Sig .000 | M203.13 Sig .061 | M999.13 Sig .000 | M1415.31 Sig .000 | M179.15 Sig .160 | M64.90 Sig .999 | M278.09 Sig .002 | M1532.22 Sig .000 | M1932.10 Sig .000 | M2177.16 Sig .000 | M1877.44 Sig .000 | M2174.72 Sig .000 |
| G5 | M1729.89 Sig .000 | M205.23 Sig .056 | M163.99 Sig .271 | M199.42 Sig .072 | | M1730.00 Sig .000 | M1786.74 Sig .000 | M402.55 Sig .000 | M1198.54 Sig .000 | M1215.89 Sig .000 | M20.26 Sig 1.0 | M264.32 Sig .003 | M1378.67 Sig .991 | M1332.80 Sig .000 | M1732.68 Sig .000 | M1977.74 Sig .000 | M1678.02 Sig .000 | M1975.31 Sig .000 |
| G6 | M.109 Sig 1.0 | M1935.24 Sig .000 | M1893.99 Sig .000 | M1929.42 Sig .000 | M1730.00 Sig .000 | | M56.74 Sig 1.0 | M2132.55 Sig .000 | M2928.55 Sig .000 | M514.11 Sig .000 | M1750.27 Sig .000 | M1994.32 Sig .000 | M1651.33 Sig .000 | M397.20 Sig .000 | M2.68 Sig 1.0 | M247.74 Sig .008 | M51.98 Sig 1.0 | M243.30 Sig .009 |
| G7 | M56.84 Sig 1.0 | M1991.97 Sig .000 | M1950.73 Sig .000 | M1986.15 Sig .000 | M1786.74 Sig .000 | M56.74 Sig 1.0 | | M2189.28 Sig .000 | M2985.28 Sig .000 | M570.85 Sig .000 | M1807.00 Sig .000 | M2051.05 Sig .000 | M1708.07 Sig .000 | M453.94 Sig .000 | M54.06 Sig 1.0 | M191.00 Sig .101 | M108.72 Sig .868 | M188.57 Sig .112 |
| G8 | M2132.44 Sig .000 | M197.31 Sig .078 | M238.56 Sig .012 | M203.13 Sig .061 | M402.55 Sig .000 | M2132.55 Sig .000 | M2189.28 Sig .000 | | M796 Sig .000 | M1618.44 Sig .000 | M382.28 Sig .000 | M138.23 Sig .548 | M481.22 Sig .000 | M1735.35 Sig .000 | M2135.23 Sig .000 | M2380.29 Sig .000 | M2080.57 Sig .000 | M2377.85 Sig .000 |
| G9 | M2928.44 | M993.31 | M1034.55 | M999.13 | M1198.54 | M2928.55 | M2985.28 | M796 | | M2414.43 | M1178.28 | M934.23 | M1277.21 | M2531.34 | M2931.22 | M3176.28 | M2876.56 | M3173.85 |

| | | | | | | | | | | | | | | | | | | |
|-----|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| | G1 | G2 | G3 | G4 | G5 | G6 | G7 | G8 | G9 | G10 | G11 | G12 | G13 | G14 | G15 | N1 | N2 | N3 |
| | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 |
| G10 | M514.00 | M1421.12 | M1379.88 | M1415.31 | M1215.89 | M514.11 | M570.85 | M1618.44 | M2414.43 | | M1236.16 | M1480.21 | M1137.22 | M116.91 | M516.79 | M761.85 | M462.13 | M759.41 |
| | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | | Sig .000 | Sig .000 | Sig .000 | Sig .794 | Sig .000 | Sig .000 | Sig .000 | Sig .000 |
| G11 | M1750.16 | M184.97 | M143.72 | M179.15 | M20.26 | M1750.27 | M1807.00 | M382.28 | M1178.28 | M1236.16 | | M244.05 | M98.93 | M1353.07 | M1752.94 | M1998.01 | M1698.29 | M1995.57 |
| | Sig .000 | Sig .129 | Sig .483 | Sig .160 | Sig 1.0 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | | Sig .009 | Sig .932 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 |
| G12 | M1994.21 | M59.08 | M100.33 | M64.90 | M264.32 | M1994.32 | M2051.05 | M138.23 | M934.23 | M1480.21 | M244.05 | | M342.99 | M1597.12 | M1997.00 | M2242.06 | M1942.34 | M2239.62 |
| | Sig .000 | Sig 1.0 | Sig .925 | Sig .999 | Sig .003 | Sig .000 | Sig .000 | Sig .548 | Sig .000 | Sig .000 | Sig .009 | | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 |
| G13 | M1651.22 | M283.90 | M242.66 | M278.09 | M78.67 | M1651.33 | M1708.07 | M481.22 | M1277.21 | M1137.22 | M98.93 | M342.99 | | M1254.13 | M1654.01 | M1899.07 | M1599.35 | M1896.64 |
| | Sig .000 | Sig .001 | Sig .010 | Sig .002 | Sig .991 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .932 | Sig .000 | | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 |
| G14 | M397.09 | M1538.03 | M1496.79 | M1532.22 | M1332.80 | M397.20 | M453.94 | M1735.35 | M2531.34 | M116.91 | M1353.07 | M1597.12 | M1254.13 | | M399.88 | M644.94 | M345.22 | M642.51 |
| | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .794 | Sig .000 | Sig .000 | Sig .000 | | Sig .000 | Sig .000 | Sig .000 | Sig .000 |
| G15 | M2.79 | M1937.91 | M1896.67 | M1932.10 | M1732.68 | M2.68 | M54.06 | M2135.23 | M2931.22 | M516.79 | M1752.94 | M1997.00 | M1654.01 | M399.88 | | M245.06 | M54.66 | M242.63 |
| | Sig 1.0 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig 1.0 | Sig 1.0 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | | Sig .009 | Sig 1.0 | Sig .010 |
| N1 | M247.85 | M2182.97 | M2141.73 | M2177.16 | M1977.74 | M247.74 | M191.00 | M2380.29 | M3176.28 | M761.85 | M1998.01 | M2242.06 | M1899.07 | M644.94 | M245.06 | | M299.72 | M2.43 |
| | Sig .008 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .008 | Sig .101 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .009 | | Sig .001 | Sig 1.0 |
| N2 | M51.87 | M1883.25 | M1842.01 | M1877.44 | M1678.02 | M51.98 | M108.72 | M2080.57 | M2876.56 | M462.13 | M1698.29 | M1942.34 | M1599.35 | M345.22 | M54.66 | M299.72 | | M297.28 |
| | Sig 1.0 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig 1.0 | Sig .868 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig 1.0 | Sig .001 | | Sig .001 |
| N3 | M245.41 | M2180.54 | M2139.30 | M2174.72 | M1975.31 | M245.30 | M188.57 | M2377.85 | M3173.85 | M759.41 | M1995.57 | M2239.62 | M1896.64 | M642.51 | M242.63 | M2.43 | M297.28 | |
| | Sig .009 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .009 | Sig .112 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .000 | Sig .010 | Sig 1.0 | Sig .001 | |

4.3 Immunofluorescence

Under normal conditions HSPA protein in cells is localized in the cytoplasm, however under conditions of stress HSPA migrates to the nucleus. Immunofluorescence detection staining was carried out utilising a monoclonal primary HSPA antibody to identify the presence and localization of HSPA protein in the NHA and the three glioma cells lines, 1321N1, GOS-3 and U87-MG for any comparable differences between normal (unstressed) and cancerous cells (stressed).

Cells cultured on chamber slides were fixed using 4% paraformaldehyde (BDH, UK) for 10 min then incubated with 0.3% Triton X-100 (BDH) in PBS for 7 min after hypoxia treatment. Slides were incubated in blocking solution containing 1% BSA (Sigma,UK) and PBS for 30 min. HSPA1A primary antibody (1:200) (Abcam, UK) was diluted in the blocking solution and applied to the cells for 1 h at room temperature. After three washes in PBS, cells were incubated with light sensitive Anti-Mouse IgG FITC conjugated secondary antibody (1:200) (Sigma, UK) diluted in blocking solution for 1 h at room temperature. All slides were washed three times in PBS and counter stained with VECTASHIELD® (1.5 µg/ml) mounting medium with Propidium iodide (PI; Vector, USA) for 10 min. Cells were visualized and scanned on an Axiovert 200M LSM 510 laser scanning confocal microscope (Carl Zeiss Ltd, UK) as described by Shervington *et al.*, (2009).

HSPA antigens detected using an Anti-mouse IgG FITC conjugated secondary antibody were predominantly identified in both the nucleus and the cytoplasm in the three glioma cell lines; 1321N1, GOS-3 and U87-MG. HSPA protein was detected in the cytoplasm

in the normal astrocyte cell line, NHA, with only limited fluorescence emitted from the nucleus of the cells. (Figures 4.5, 4.6, 4.7 and 4.8).

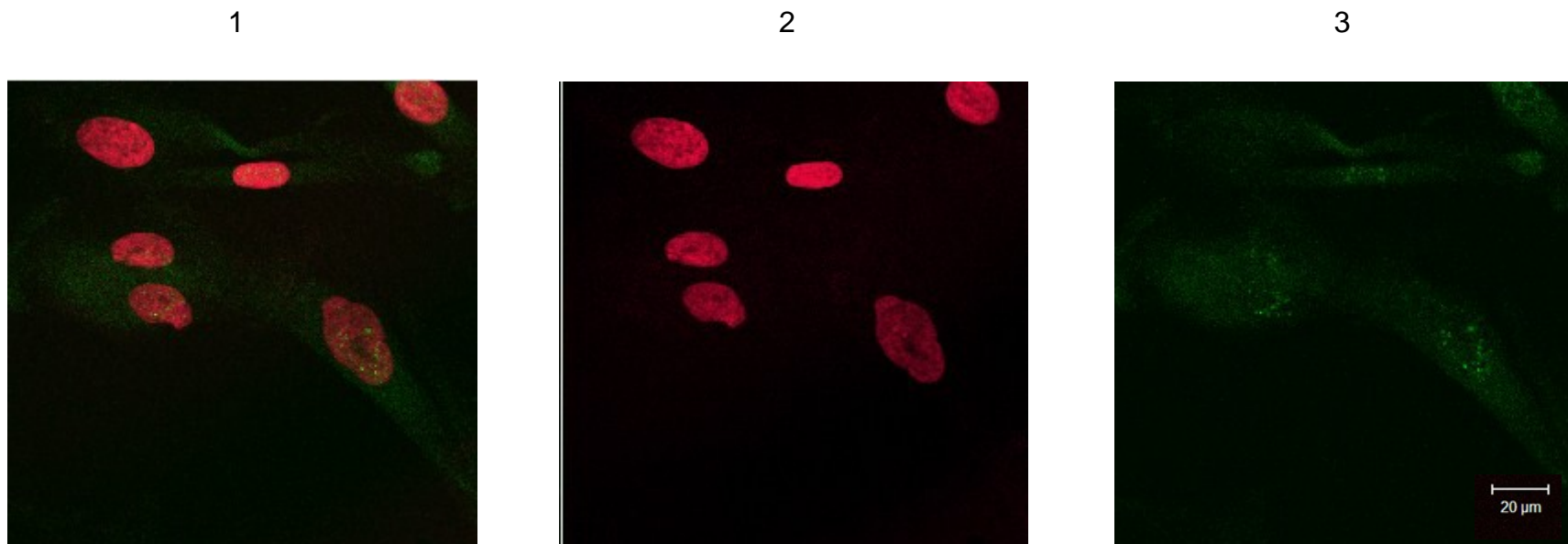


Figure 4.5 HSPA protein levels assessed using immunofluorescence in untreated NHA cells. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

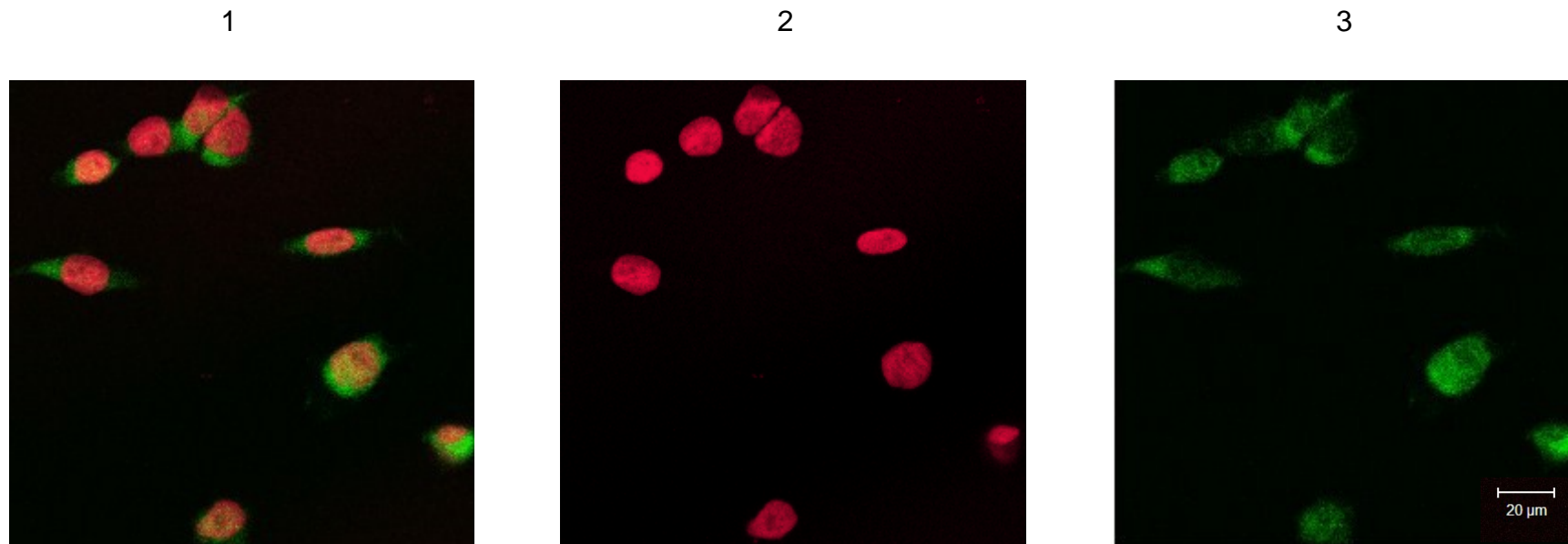


Figure 4.6 HSPA protein levels assessed using immunofluorescence in untreated 1321N1 cells. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

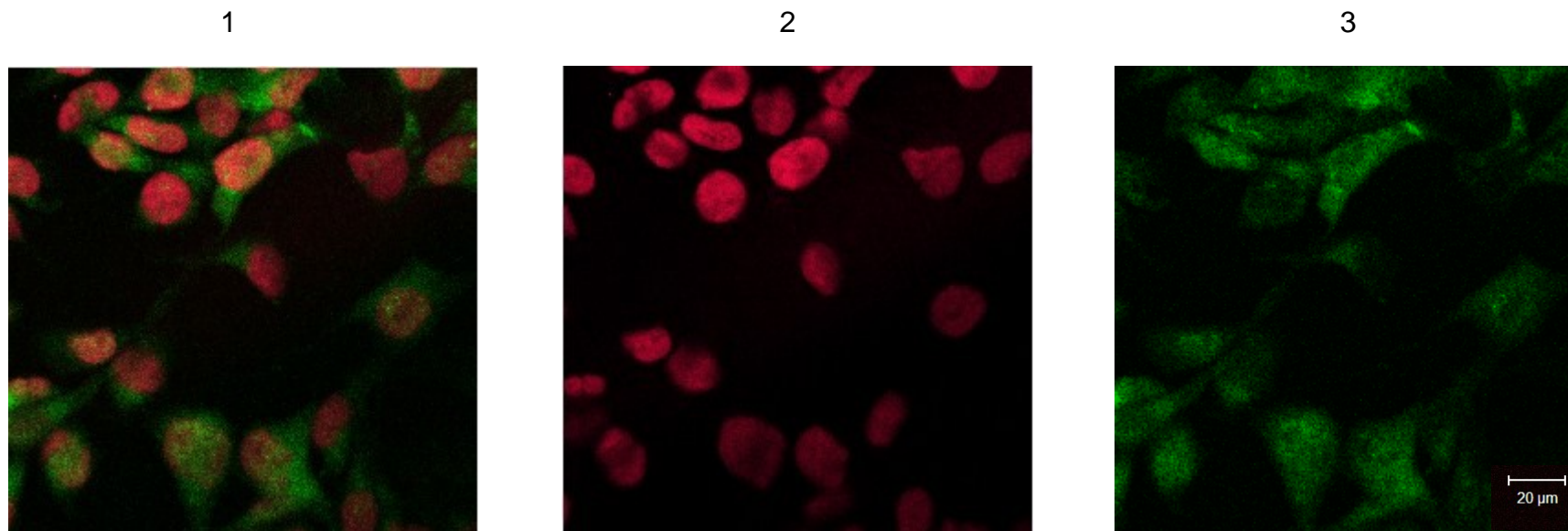


Figure 4.7 HSPA protein levels assessed using immunofluorescence in untreated GOS-3 cells. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

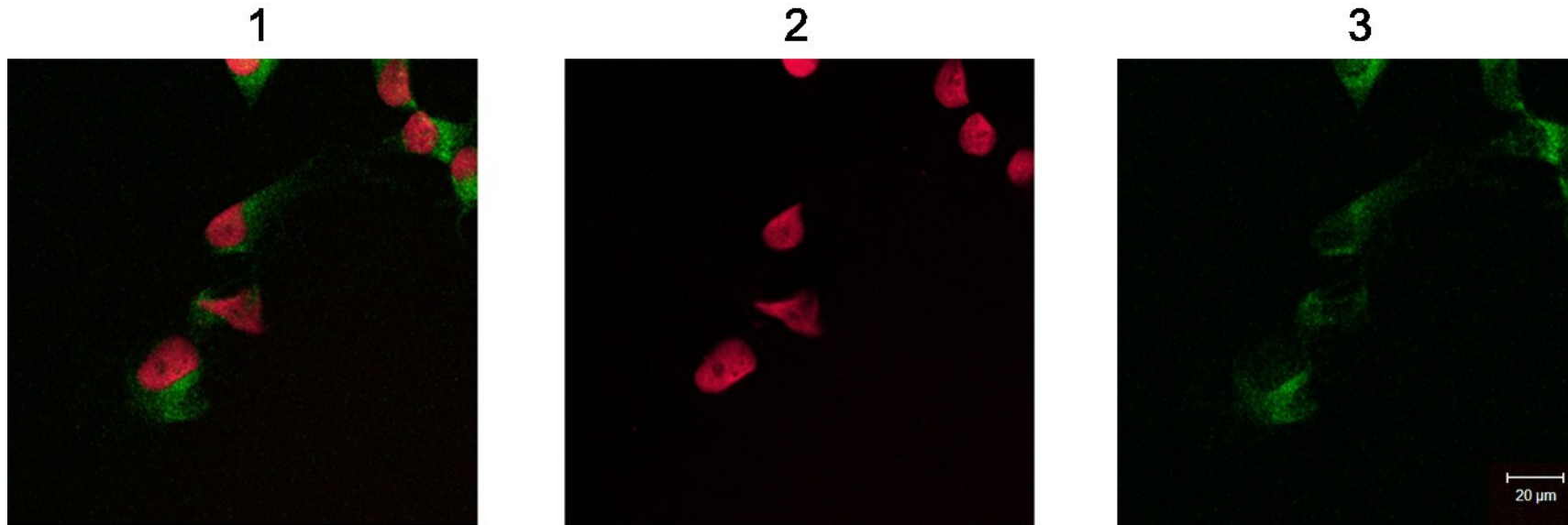


Figure 4.8 HSPA protein levels assessed using immunofluorescence in untreated U87-MG cells. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20µm.

4.4 Flow Cytometry

To accurately quantify the presence of HSPA protein, flow cytometry analysis was carried out. The data obtained from flow cytometry was used to produce fluorescence intensity histograms, which depict the distribution of cell surface antigen densities within the population under study, and provide a molecular fingerprint of protein distribution. Comparisons of HSPA protein production were made between the different cell lines.

Flow cytometry involves the use of fluorescent labelled antibodies and fluorescent detectors to analyse large numbers of cells sequentially. Antibodies bind to their respective target molecule or antigen in a one-to-one ratio, such that the number of antibodies bound to a cell and the number of fluorescent molecules present, will in general be proportional to the level of production of that protein on the cell.

Cultured cells were stained and subjected to flow cytometry, briefly 1×10^6 were washed once in 0.1% BSA in PBS before being re-suspended in 0.1% Triton X-100 for 15 min. Following incubation cells were collected and washed once with 0.1% BSA in PBS before being blocked in 5% goat serum in 0.1% BSA in PBS for 30 min. HSPA primary antibody (1:200) diluted in blocking solution was applied for 30 min. Cells were then washed with 0.1% BSA in PBS and light sensitive Anti-Mouse IgG FITC conjugated secondary antibody (1:200) diluted in blocking solution was added for 30 min. Following incubation cells were washed twice in 0.1% BSA in PBS before finally being re-suspended in an aliquot 0.1% BSA in PBS and filtered into FACS tubes. All incubation steps were performed on ice.

As expected, analysis showed high relative fluorescence intensity, with approximately 98% of the 1321N1 cell population showing positive, approximately 89% of GOS-3 cell population showing positive, and 97% of the U87-MG cell population showing positive compared to little or no fluorescence intensity in the NHA cell line, approximately 0.13% showing positive for HSPA. This is taken to indicate that HSPA protein is barely detectable in normal cells, but is present at significantly higher levels in cancer cells; approximately 97-fold higher in 1321N1, 89-fold higher in GOS-3 and 97-fold higher in U87-MG (Figures 4.9, 4.10, 4.11, 4.12 and 4.13).

A

| Cell Line | Total % of cells +ve for HSPA |
|-----------|-------------------------------|
| NHA | 0 |
| 1321N1 | 97.1 |
| GOS-3 | 89.2 |
| U87-MG | 96.9 |

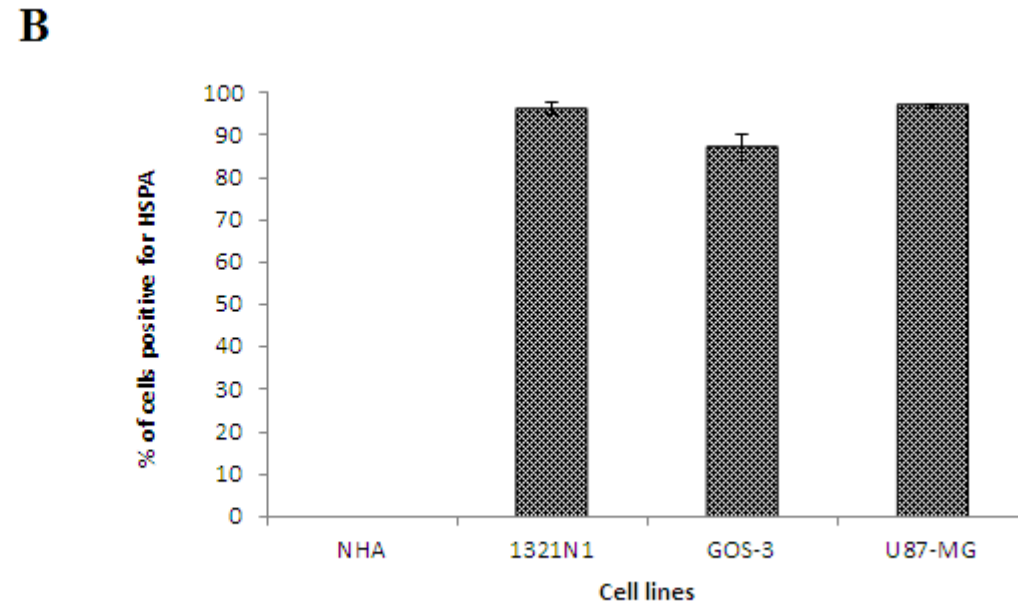


Figure 4.9 Average percentages of cells showing positive for HSPA. (A) Average percentage of normal and glioma cells positive for HSPA protein detected by flow cytometry, considering 10,000 events per sample. (B) Histogram showing the mean percentage of cells evaluated by flow cytometry of three independent experiments, and the standard deviation from this mean is shown by the error bars. No column was shown on the histogram for NHA as the value = 0.

4.4.1 Statistical Analysis

A one-way ANOVA test between groups of analysis of variance was conducted to access HSPA protein production in normal and glioma cell lines in three independent experiments. The cell lines comprised of four groups: group 1 NHA, group 2 1321N1, group 3 GOS-3 and group 4 U87-MG. The means and standard deviations are presented in Table 4.8.

Table 4.8 Descriptive statistics showing the mean for HSPA protein levels for each cell line.

| Cell Line | N | Mean % of HSPA protein levels | Std Deviation |
|-----------|---|-------------------------------|---------------|
| NHA | 3 | 0.133 | 0.057 |
| 1321N1 | 3 | 96.87 | 0.321 |
| GOS-3 | 3 | 89.15 | 0.050 |
| U87-MG | 3 | 96.85 | 0.050 |

There was significant difference in HSPA protein levels for the four cell line groups [$F(3,8) = 239590$, $P < 0.001$]. The effect size, calculated using eta squared indicated a large effect of 0.999.

Table 4.9. Statistical data from Tukey's HSD test showing significance of HSPA protein levels between cell lines. Any significant differences $P \leq 0.05$ are highlighted in bold.

| (I) Cell | J (Cell) | % Mean Difference (I – J) | Std. Error | Sig. |
|---------------|---------------|---------------------------|--------------|--------------|
| NHA | 1321N1 | 96.73 | 0.136 | 0.000 |
| NHA | GOS-3 | 89.02 | 0.136 | 0.000 |
| NHA | U87-MG | 96.72 | 0.136 | 0.000 |
| 1321N1 | GOS-3 | 7.72 | 0.136 | 0.000 |
| 1321N1 | U87-MG | 0.0168 | 0.136 | 0.999 |
| GOS-3 | U87-MG | 7.70 | 0.136 | 0.000 |

Post-hoc analyses using Tukey's HSD test indicated significantly lower HSPA protein levels in group 1, NHA than in: group 2, 1321N1 (MD = 96.73, $P < .05$); group 3, GOS-3 (MD = 89.02, $P < .05$) and group 4, U87-MG (MD = 96.72, $P < .05$) (Table 4.9).

There was a significantly lower HSPA protein levels in group 2, 1321N1 than in group 3, GOS-3 (MD = 7.72, $P < .05$) (Table 4.9).

There was a significantly lower HSPA protein levels in group 3, GOS-3 than in group 4, U87-MG (MD = 7.70, $P < .05$) (Table 4.9).

All other differences between groups failed to reach significance (Table 4.9).

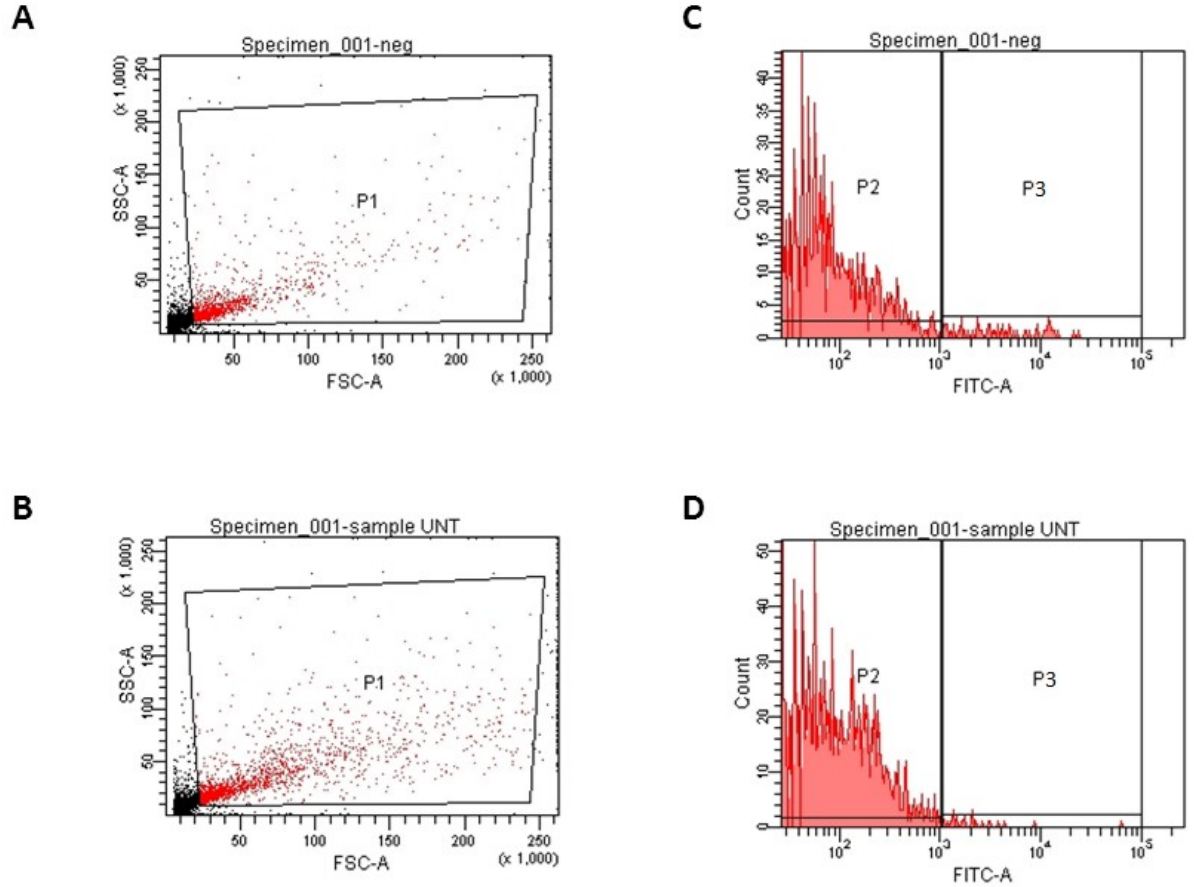


Figure 4.10 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the NHA cell lines. No fluorescence intensity was observed in the NHA cell line (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

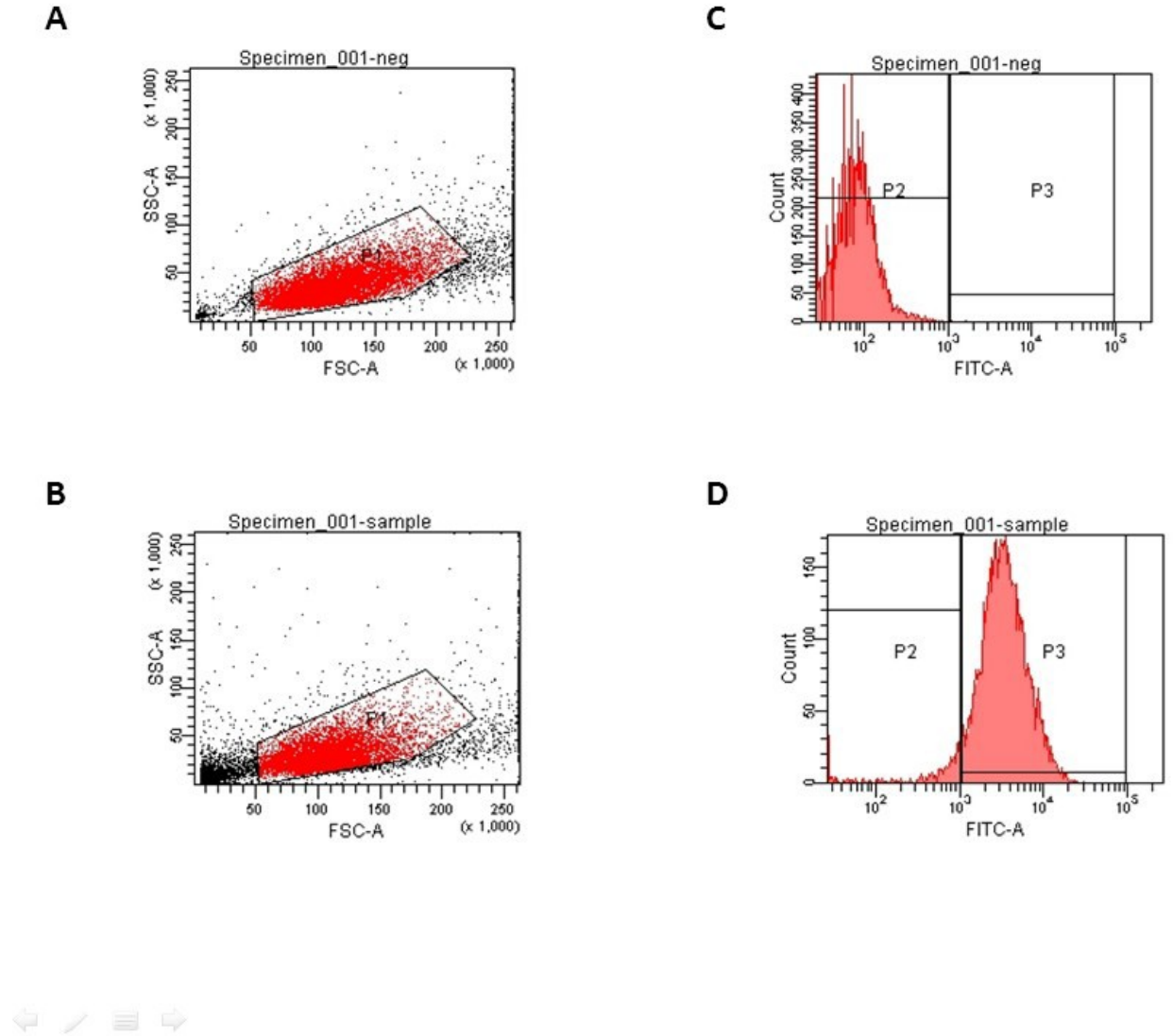


Figure 4.11 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the 1321N1 cell lines. Approximately 97% of 1321N1 glioma cells showed positive for HSPA. (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

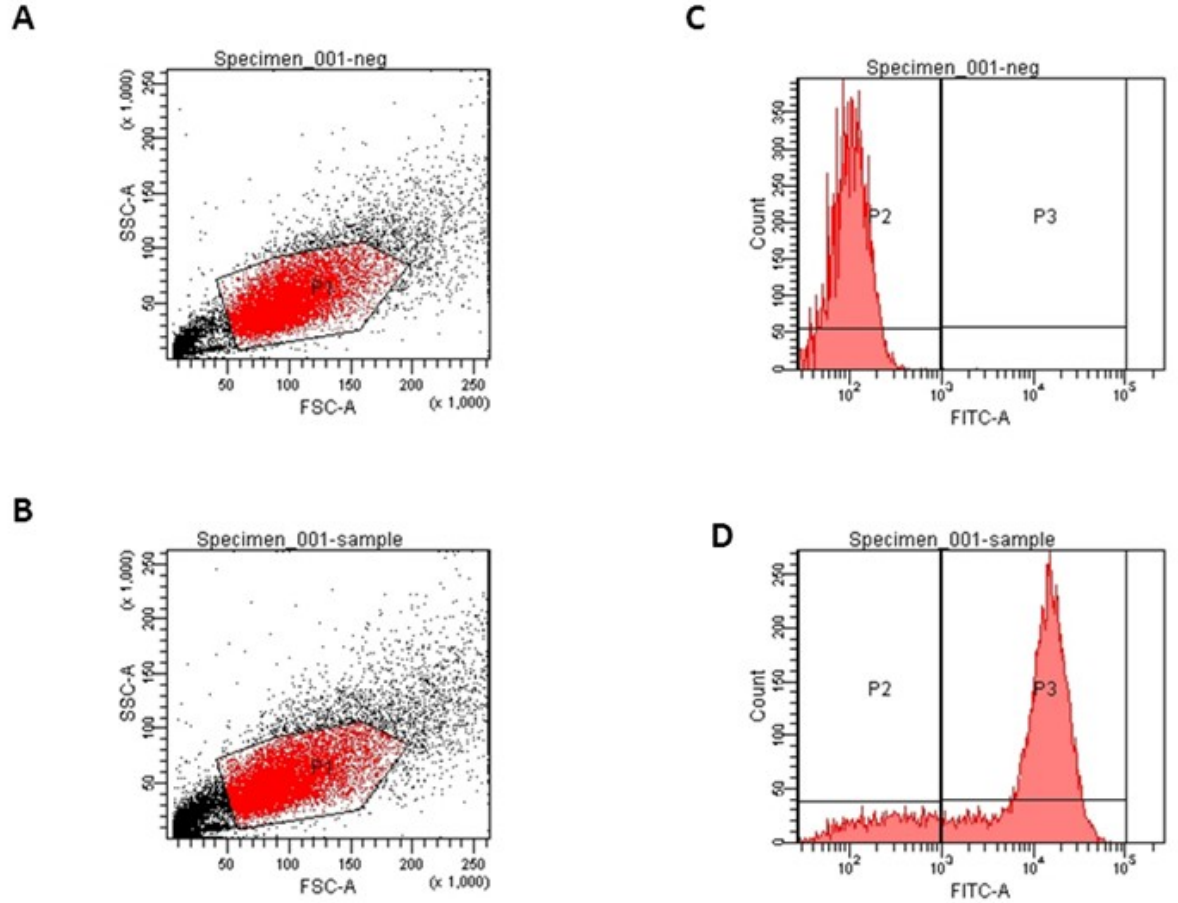


Figure 4.12 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the GOS-3 cell lines. Approximately 89% of GOS-3 glioma cells showed positive for HSPA. (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

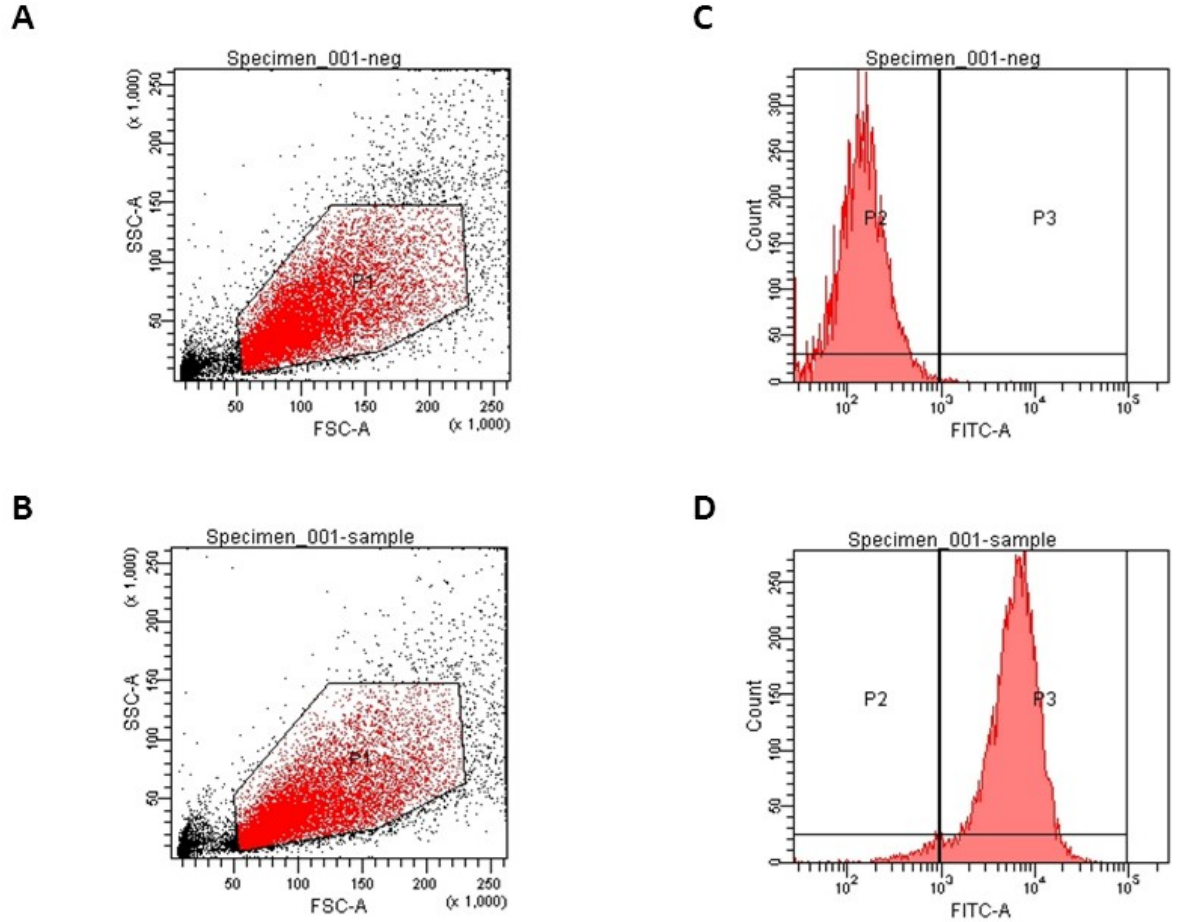


Figure 4.13 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the U87-MG cell lines. Approximately 97% of U87-MG glioma cells showed positive for HSPA. (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

Taken together, the results from these experiments show that *HSPA* is expressed at low levels in normal cells and normal brain tissue, but is more highly expressed in cancer cell lines and brain tumours. The average *HSPA* mRNA copy numbers in the three glioma cell lines were approximately 6 fold higher than the normal cell line, and the average *HSPA* mRNA copy numbers in glioblastoma tissue were approximately 1.8 and 9 fold higher than the low grade glioma and normal tissue respectively, suggesting a grade-related transcription profile.

As with *HSPA* mRNA, high levels of HSPA protein were detected in the three glioma cell lines compared to low/barely detectable levels in the NHA cell line, indicating a direct correlation between *HSPA* mRNA and HSPA protein levels in glioma cell lines.

These results suggest that *HSPA* mRNA and HSPA protein levels in glioma may possibly be grade related, and therefore may be useful as a possible prognostic marker.

As expected, given that cancer is a form of cellular stress, HSPA protein was detected in both the cytoplasm and the nucleus of the three glioma cell lines. HSPA protein was detected predominantly in the cytoplasm in the normal astrocyte cell line, NHA with only limited fluorescence emitted from the nucleus of the cells.

Chapter 5

Effects of hypoxia on *HSPA* gene expression in glioma

5.1 Gene Expression

HSPA expression is up-regulated in cells by cancer and also in response to hypoxia, and the main research aim of this chapter was to measure and compare *HSPA* expression in normal and glioma cell lines under pre- and post-hypoxic conditions, with a view to characterising the expression of *HSPA*. The potential correlation between *HSPA* expression and hypoxia was investigated using three glioma cell lines (1321N1, GOS-3 and U87-MG) and the normal human astrocyte cell line (NHA). Expression of *HSPA* was compared between the three glioma cell lines (1321N1, GOS-3 and U87-MG) and with a normal (control) human astrocyte cell line (NHA), under pre- and post-hypoxia conditions. *GAPDH*, was used as an internal standard throughout all RT-PCR experiments (Barber *et al*, 2005).

Human brain cell lines GOS-3 (grade II/III oligodendroglioma) from DMSZ (Germany), U87-MG (grade IV glioblastoma) from ECCAC (UK) and NHA (normal human astrocytes) from Lonza (UK) were used in this study. GOS-3 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% FBS and with 4 mM L-glutamide, while U87-MG cells were cultured in Eagle's minimum essential medium (EMEM) (Sigma) supplemented with 2 mM L-glutamide, 10% FBS and 1% (v/v) nonessential amino acids (Sigma). The NHA cells were cultured in astrocyte medium (AM) supplemented with 15 ml of FBS, 0.5 ml Ascorbic Acid, 0.5 ml rhEGF (astrocyte growth supplement) 0.5 ml GA100 1.25 ml Insulin and 5 ml L-glutamide.

Hypoxic conditions were induced by exposing confluent cells to nitrogen (100 %) for 30 min (Kay *et al*, 2007). After treatment, the cells were collected at various recovery

periods (0, 3, 6 and 24 h) for experiments concerning gene expression, immunofluorescence and flow cytometry.

mRNA was isolated from all four cell lines, using mRNA Isolation Kit (Roche,UK) following the manufacturer's protocol. The concentration and purity of mRNA was determined by ultraviolet spectrophotometry. Isolated mRNA (100 ng) was transcribed to cDNA using 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche, UK) following the manufacturer's protocol, which was then used as a template for qRT-PCR. Quantitative real-time PCR was used to evaluate the expression of *HSPA* and *GAPDH* as a control using FastStart DNA Master^{PLUS} SYBR Green 1 (Roche, UK).

Primers used for *HSPA* were 5' CGACCTGAACAAGAGCATCA 3' (sense) and 5' AAGATCTGCGTCTGCTTGGT 3' (antisense). For *GAPDH*, primers were 5' GAGTCAAGCGATTTGGTCGT 3' (sense) and 5' TTGATTTTGGAGGGATCTCG 3' (antisense). All primers were designed using Primer3 software and manufactured by TIB MOLBIOL.

After an initial denaturation at 95°C for 10 min, the samples were subjected to 35 cycles of RT-PCR 95°C for 10 s, annealing temperature 57°C (*HSPA*) and 56°C (*GAPDH*) for 15 s, and 72°C for 15 s (Patel *et al.*, 2008). At the end of each cycle, the fluorescence emitted was measured in a single step in channel F1 (gain1). After the 35th cycle, the specimens were heated to 95°C and rapidly cooled to 65°C for 15 s. All heating and cooling steps were performed with a slope of 20°C / s. The temperature was subsequently raised to 95°C with a slope of 0.1°C / s and fluorescence was measured continuously (channel F1, gain1) to obtain data for the melting curve analysis. All PCR reactions were performed in triplicate and a negative control included, which contained primers with no DNA. All PCR products were analysed using gel electrophoresis stained and visualised using a gel analyser (SynGene, UK).

5.2 Constitutive Expression

5.2.1 Glioma Cell lines

Transcript copy numbers of *HSPA* and *GAPDH* were determined using RT-PCR for three glioma cell lines; 1321N1, GOS-3 and U87-MG and in the normal human astrocyte cell line NHA, under pre- and post-hypoxia treatment. The primers and optimal temperatures used for the amplification of both genes are documented in Table 2.10 in section 2.5. All PCR experiments were carried out in triplicate for consistency and repeatability. For each gene analysed, a quantification graph was produced to confirm gene amplification. The resulting amplicons for *HSPA* and *GAPDH* were then visualized using agarose gel electrophoresis, each being represented by bands of 213 and 238 bp respectively.

The mRNA copy number per 100 ng of extracted mRNA for both genes was calculated for each cell line. High levels of *HSPA* mRNA expression were observed in 1321N1, GOS-3 and U87-MG glioma cell lines pre-hypoxia treatment, with approximate copy numbers of each of 7,500, 8,900, 8,200 respectively and post-hypoxia treatment after 0 h recovery, with approximate copy numbers of each of 8,200, 7,300, 8,100 respectively; after 3 h recovery, with approximate copy numbers of each of 8100, 7,500, 7,800 respectively; after 6 h recovery, with approximate copy numbers of each of 8,100, 7,500, 8,200 respectively and after 24 h recovery, with approximate copy numbers of each of 7,800, 7,800, 8,200 respectively (Figure 5.1 and Table 5.1). In contrast, the normal human astrocyte cell line showed low but detectable levels of *HSPA* mRNA in pre hypoxia treatment, with an approximate copy number of 1,400 and slightly raised levels of *HSPA* mRNA in post hypoxia treatment after 0 h recovery, with an approximate copy number of 2,500, after 3 h recovery, with an approximate copy

number of 1,100, after 6 h recovery, with an approximate copy number of 590 and after 24 h recovery, with an approximate copy number of 350 (Figure 5.1 and Table 5.1).

For *GAPDH*, mRNA copy numbers per 100 ng of extracted mRNA were relatively consistent for NHA, with approximate copy numbers of 148,300, for 1321N1, with approximate copy numbers of 147,400, for GOS-3, with approximate copy numbers of 147,400 and for U87-MG, with approximate copy numbers of 147,300, again confirming comparability of HSPA results (Figure 5.2 and Table 5.1).

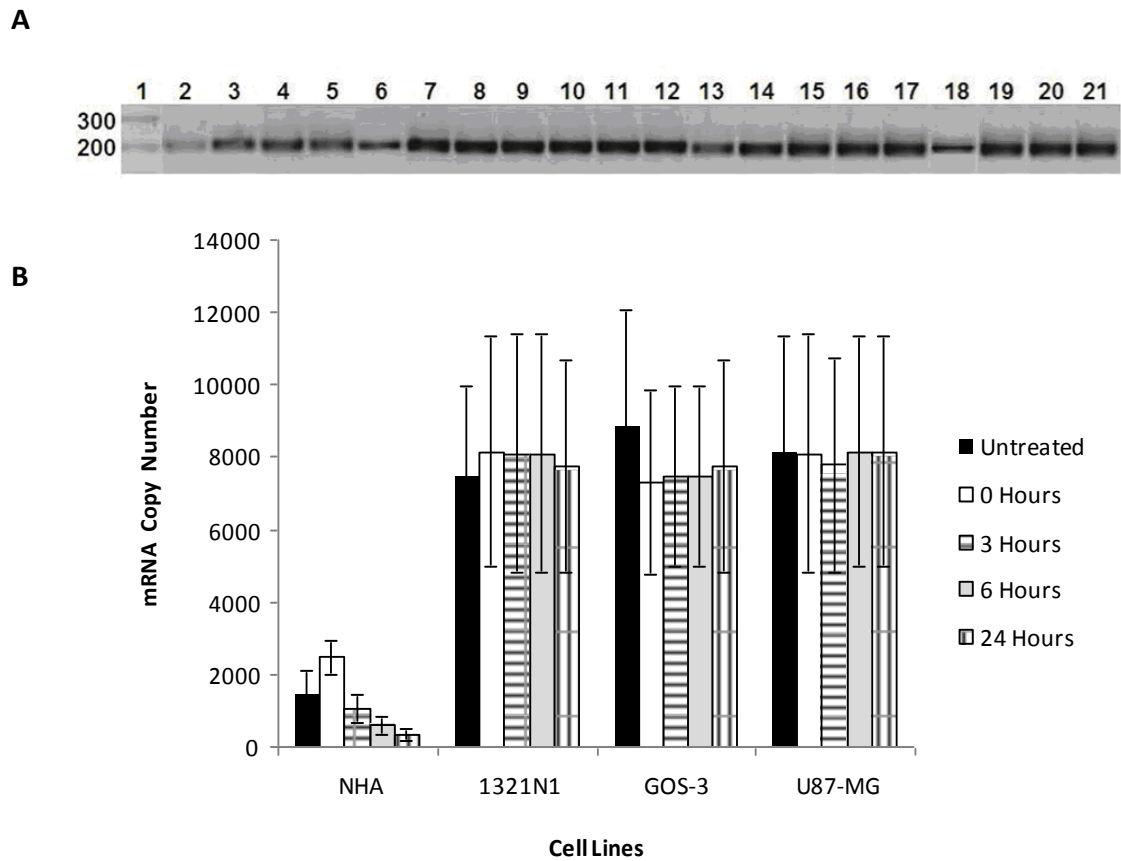


Figure 5.1 Levels of *HSPA* mRNA transcripts in pre- and post-hypoxia treated NHA, 1321N1, GOS-3 and U87-MG cells. (A) agarose gel electrophoresis: Lane 1 represents the 100 bp molecular ladder; lanes 2 - 6 represent amplicons from NHA: pre-hypoxia, post-hypoxia after 0, 3, 6 and 24 h recovery, lanes 7 - 11 represent amplicons from 1321N1: pre-hypoxia, post-hypoxia after 0, 3, 6 and 24 h recovery, lanes 12 - 16 represent amplicons from GOS-3: pre-hypoxia, post-hypoxia after 0, 3, 6 and 24 h recovery and lanes 17 - 21 represent amplicons from U87-MG: pre-hypoxia, post-hypoxia after 0, 3, 6 and 24 h recovery. (B) Histogram showing *HSPA* mRNA copy numbers per 100 ng of extracted mRNA for pre and post hypoxia 0, 3, 6 and 24 h, treated cell lines. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars.

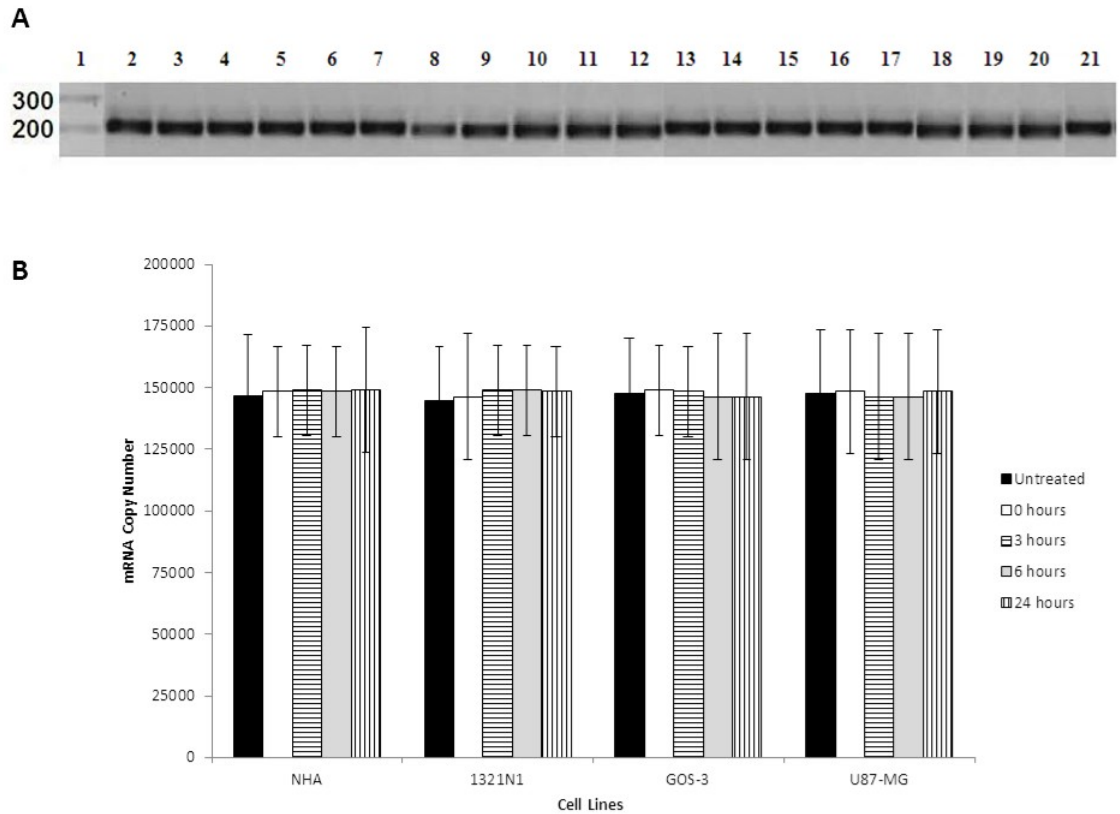


Figure 5.2 Levels of *GAPDH* mRNA transcripts in pre and post hypoxia treated NHA, 1321N1, GOS-3 and U87-MG cells. (A) agarose gel electrophoresis: Lane 1 represents the 100 bp molecular ladder; lanes 2 - 6 represent amplicons from NHA: pre-hypoxia, post-hypoxia after 0, 3, 6 and 24 h recovery, lanes 7 - 11 represent amplicons from 1321N1: pre-hypoxia, post-hypoxia after 0, 3, 6 and 24 h recovery, lanes 12 - 16 represent amplicons from GOS-3: pre-hypoxia, post-hypoxia after 0, 3, 6 and 24 h recovery and lanes 17 - 21 represent amplicons from U87-MG: pre-hypoxia, post-hypoxia after 0, 3, 6 and 24 h recovery. (B) Histogram showing *HSPA* mRNA copy numbers per 100 ng of extracted mRNA for pre and post hypoxia 0, 3, 6 and 24 h, treated cell lines. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars.

5.2.2 Statistical Analysis

A two-way factorial mixed 4 (cell line: NHA, 1321N1, GOS-3, U87-MG) x 5 (treatment: untreated, 0, 3, 6 and 24 h recovery after hypoxia treatment) ANOVA analysis of variance test was performed to investigate differences of *HSPA* and *GAPDH* gene expression. The means and standard deviation are presented in Table 5.1.

For *HSPA* gene expression there was a non-significant effect for: cell line [$F(15,11.44) = .832$, $P = 0.637$]; Wilks' Lambda = 0.131; partial eta squared = 0.493 and treatment [$F(4,5) = 1.692$; $P = 0.287$], Wilks' Lambda = 0.425, partial eta squared = 0.575. The interaction between treatment and cell line also failed to reach significance [$F(12,13.52) = 1.0$; $P = 0.496$], Wilks' Lambda = 0.186, partial eta squared = 0.429.

There was a statistically significant main effect in *HSPA* transcript copy numbers for cell lines on the combined variables: [$F(3,8) = 6.544$, $P = .015$]; partial eta squared (η_p^2) = 0.710.

When the results for the dependent variables were considered separately, there was a statistical significance in untreated [$F(3,8) = 5.157$, $P = .028$]; partial eta squared (η_p^2) = 0.659.

Post-hoc analyses using Tukey's HSD test indicated significantly lower *HSPA* transcript copy numbers in untreated NHA than in untreated, GOS-3 (MD = 7459.67, $P = 0.033$). All other differences between groups failed to reach significance (Table 5.2).

There was a non statistical significance after 0 h recovery [$F(3,8) = 3.187$, $P = .084$]; partial eta squared (η_p^2) = 0.544. Posthoc analyses using Tukey's HSD indicated that all differences between groups failed to reach significance (Table 5.2)

There was a statistical significance after 3 h recovery [$F(3,8) = 5.260$, $P = .027$]; partial eta squared (η_p^2) = 0.664. Posthoc analyses using Tukey's HSD indicated significantly

lower *HSPA* transcript copy numbers after 3 h recovery in NHA than in: 1321N1 (MD 7042.05, $P < .05$) and U87-MG (MD = 6730.03, $P < .05$). All other differences between groups failed to reach significance (Table 5.2).

There was a statistical significance after 6 h recovery [$F(3,8) = 5.910$, $P = .020$]; partial eta squared (η_p^2) = 0.689. Posthoc analyses using Tukey's HSD indicated significantly lower *HSPA* transcript copy numbers after 6 h recovery in NHA than in: 1321N1 (MD 7510.99, $P < .05$); GOS-3 (MD = 6901.49, $P < .05$) and U87-MG (MD = 7567.85, $P < .05$). All other differences between groups failed to reach significance (Table 5.2).

There was a statistical significance after 24 h recovery [$F(3,8) = 6.232$, $P = .017$]; partial eta squared (η_p^2) = 0.700.

Posthoc analyses using Tukey's HSD indicated significantly lower *HSPA* transcript copy numbers after 24 h recovery in NHA than in: 1321N1 (MD 7414.30, $P < .05$); GOS-3 (MD = 7414.30, $P < .05$) and U87-MG (MD = 7807.93, $P < .05$). All other differences between groups failed to reach significance (Table 5.2).

For *GAPDH* transcript copy numbers, there was a non significant effect for: cell line [$F(15,11.44) = 0.005$, $P > .05$]; Wilks' Lambda = 0.980, partial eta squared = 0.007 and treatment [$F(4,5) = .003$; $P > .05$], Wilks' Lambda = 0.998, partial eta squared = 0.002. The interaction between treatment and cell line also failed to reach significance [$F(12,13.52) = .007$; $P > .05$], Wilks' Lambda = .983, partial eta squared = 0.006.

There was a non significant main effect in *GAPDH* gene expression for cell lines on the combined variables: [$F(3,8) = .001$, $P > .05$]; partial eta squared (η_p^2) = 0.000.

When the results for the dependent variables were considered separately, there was a non statistical significance in untreated [$F(3,8) = .004$, $P > .05$]; partial eta squared (η_p^2)

= 0.002. Posthoc analyses using Tukey's HSD indicated that all differences between groups failed to reach significance.

There was a non statistical significance after 0 h recovery [$F(3,8) = .005$, $P > .05$]; partial eta squared (η_p^2) = 0.002. Posthoc analyses using Tukey's HSD indicated that all differences between groups failed to reach significance.

There was a non statistical significance after 3 h recovery [$F(3,8) = .004$, $P > .05$]; partial eta squared (η_p^2) = 0.002. Posthoc analyses using Tukey's HSD indicated that all differences between groups failed to reach significance.

There was a non statistical significance after 6 h recovery [$F(3,8) = .007$, $P > .05$]; partial eta squared (η_p^2) = 0.003. Posthoc analyses using Tukey's HSD indicated that all differences between groups failed to reach significance.

There was a non statistical significance after 24 h recovery [$F(3,8) = .005$, $P > .05$]; partial eta squared (η_p^2) = 0.002. Posthoc analyses using Tukey's HSD indicated that all differences between groups failed to reach significance.

Table 5.1. Descriptive statistics showing the mean mRNA copy numbers for *HSPA* and *GAPDH* gene expression for untreated and treated cell lines.

| Treatment | Cell Line | N | Mean <i>HSPA</i> transcript copy number | STD. Dev. | Mean <i>GAPDH</i> Transcript copy number | STD. Dev. |
|---------------------------------------|-----------|---|---|-----------|--|-----------|
| Pre-hypoxia | NHA | 3 | 1419.63 | 708.76 | 146468.91 | 50836.00 |
| | 1321N1 | 3 | 7491.67 | 2495.20 | 144546.62 | 32831.75 |
| | GOS-3 | 3 | 8879.29 | 3210.84 | 147541.72 | 33859.90 |
| | U87-MG | 3 | 8158.12 | 3204.01 | 147541.72 | 33859.90 |
| 0 h recovery after hypoxia treatment | NHA | 3 | 2463.77 | 488.26 | 148473.30 | 18197.56 |
| | 1321N1 | 3 | 8158.12 | 3204.01 | 146112.84 | 25188.06 |
| | GOS-3 | 3 | 7286.28 | 2548.80 | 148927.19 | 46355.40 |
| | U87-MG | 3 | 8101.26 | 3299.79 | 148439.75 | 24974.58 |
| 3 h recovery after hypoxia treatment | NHA | 3 | 1059.21 | 401.35 | 148927.19 | 46355.40 |
| | 1321N1 | 3 | 8101.26 | 3299.79 | 148927.19 | 46355.40 |
| | GOS-3 | 3 | 7491.67 | 2495.20 | 148473.30 | 18197.56 |
| | U87-MG | 3 | 7789.23 | 2955.70 | 146112.84 | 25188.06 |
| 6 h recovery after hypoxia treatment | NHA | 3 | 590.27 | 233.87 | 148473.30 | 18197.56 |
| | 1321N1 | 3 | 8101.26 | 3299.79 | 148927.19 | 46355.40 |
| | GOS-3 | 3 | 7491.67 | 2495.20 | 146112.84 | 25188.06 |
| | U87-MG | 3 | 8158.12 | 3204.01 | 146112.84 | 25188.04 |
| 24 h recovery after hypoxia treatment | NHA | 3 | 350.19 | 167.09 | 148927.19 | 46355.40 |
| | 1321N1 | 3 | 7764.50 | 2930.96 | 148473.30 | 18197.56 |
| | GOS-3 | 3 | 7764.50 | 2930.96 | 146112.84 | 25188.06 |
| | U87-MG | 3 | 8158.12 | 3204.01 | 148439.75 | 24974.58 |

Overall inspection of the mean scores for *HSPA* transcript copy numbers indicated that untreated and treated 1321N1; GOS-3 and U87-MG cell lines were considerably higher than the NHA cell line.

Overall inspection of the mean scores for *GAPDH* transcript copy numbers indicated no significant differences in untreated and treated NHA, 1321N1, GOS-3 and U87-MG cell lines.

Table 5.2. Statistical data from Tukey's HSD test showing significance of *HSPA* transcript copy numbers in treated and untreated cell lines. Any significant differences are highlighted in bold. Significance ≤ 0.05 .

| | | NHA | | 1321N1 | | GOS-3 | | U87-MG | |
|---------------------------------------|-----------|---|-------------|---|-------------|---|-------------|---|-------------|
| Treatment | Cell Line | Mean Diff \pm STD error | SIG. | Mean Diff \pm STD error | SIG. | Mean Diff \pm STD error | SIG. | Mean Diff \pm STD error | SIG. |
| Pre-hypoxia | NHA | | | 6072.04 \pm 2311.21 | .083 | 7459.67 \pm 2133.21 | .033 | 6738.49 \pm 2133.21 | .053 |
| | 1321N1 | 6072.04 \pm 2133.21 | .083 | | | 1387.62 \pm 2311.21 | .912 | 666.45 \pm 2311.21 | .989 |
| | GOS-3 | 7459.67 \pm 2133.21 | .033 | 1387.62 \pm 2311.21 | .912 | | | 721.17 \pm 2311.21 | .986 |
| | U87-MG | 6738.49 \pm 2133.21 | .053 | 666.45 \pm 2311.21 | .989 | 721.17 \pm 2311.21 | .986 | | |
| 0 h recovery after hypoxia treatment | NHA | | | 5694.35 \pm 2155.96 | .110 | 4822.51 \pm 2155.96 | .193 | 5637.49 \pm 2155.96 | .114 |
| | 1321N1 | 5694.35 \pm 2155.96 | .110 | | | 871.84 \pm 2155.96 | .976 | 56.86 \pm 2155.96 | 1.000 |
| | GOS-3 | 4822.51 \pm 2155.96 | .193 | 871.84 \pm 2155.96 | .976 | | | 814.98 \pm 2155.96 | .980 |
| | U87-MG | 5637.49 \pm 2155.96 | .114 | 56.86 \pm 2155.96 | 1.000 | 814.98 \pm 2155.96 | .980 | | |
| 3 h recovery after hypoxia treatment | NHA | | | 7042.05 \pm 2082.14 | .039 | 6432.46 \pm 2082.14 | .059 | 6730.03 \pm 2082.14 | .048 |
| | 1321N1 | 7042.05 \pm 2082.14 | .039 | | | 609.59 \pm 2082.14 | .991 | 312.02 \pm 2082.14 | .999 |
| | GOS-3 | 6432.46 \pm 2082.14 | .059 | 609.59 \pm 2082.14 | .991 | | | 297.57 \pm 2082.14 | .999 |
| | U87-MG | 6730.03 \pm 2082.14 | .048 | 312.02 \pm 2082.14 | .999 | 297.57 \pm 2082.14 | .999 | | |
| 6 h recovery after hypoxia treatment | NHA | | | 7510.99 \pm 2138.34 | .032 | 6901.40 \pm 2138.34 | .048 | 7567.85 \pm 2138.34 | .031 |
| | 1321N1 | 7510.99 \pm 2138.34 | .032 | | | 609.59 \pm 2138.34 | .991 | 56.86 \pm 2138.34 | 1.000 |
| | GOS-3 | 6901.40 \pm 2138.34 | .048 | 609.59 \pm 2138.34 | .991 | | | 666.45 \pm 2138.34 | .989 |
| | U87-MG | 7567.85 \pm 2138.34 | .031 | 56.86 \pm 2138.34 | 1.000 | 666.45 \pm 2138.34 | .989 | | |
| 24 h recovery after hypoxia treatment | NHA | | | 7414.30 \pm 2139.86 | .035 | 7414.30 \pm 2139.86 | .035 | 7807.93 \pm 2139.86 | .027 |
| | 1321N1 | 7414.30 \pm 2139.86 | .035 | | | 0.0 \pm 2139.86 | 1.000 | 393.62 \pm 2139.86 | .998 |
| | GOS-3 | 7414.30 \pm 2139.86 | .035 | 1.0 \pm 2139.86 | 1.000 | | | 393.62 \pm 2139.86 | .998 |
| | U87-MG | 7807.93 \pm 2139.86 | .027 | 393.62 \pm 2139.86 | .998 | 393.62 \pm 2139.86 | .998 | | |

5.3 Immunofluorescence

Under normal conditions HSPA protein in cells is localized in the cytoplasm, however under conditions of stress HSPA migrates to the nucleus. Immunofluorescence detection staining was carried out utilising a monoclonal primary HSPA antibody to identify the presence and localization of HSPA protein in the NHA and the three glioma cells lines, 1321N1, GOS-3 and U87-MG for any comparable differences between normal (unstressed) and cancerous cells (stressed) under pre and post hypoxic conditions.

Cells cultured on chamber slides were fixed using 4% paraformaldehyde (BDH, UK) for 10 min then incubated with 0.3% Triton X-100 (BDH) in PBS for 7 min after hypoxia treatment. Slides were incubated in blocking solution containing 1% BSA (Sigma,UK) and PBS for 30 min. HSPA primary antibody (1:200) (Abcam, UK) was diluted in the blocking solution and applied to the cells for 1 h at room temperature. After three washes in PBS, cells were incubated with light sensitive Anti-Mouse IgG FITC conjugated secondary antibody (1:200) (Sigma, UK) diluted in blocking solution for 1 h at room temperature. All slides were washed three times in PBS and counter stained with VECTASHIELD® (1.5 µg/ml) mounting medium with Propidium iodide (PI; Vector, USA) for 10 min. Cells were visualized and scanned on an Axiovert 200M LSM 510 laser scanning confocal microscope (Carl Zeiss Ltd, UK) as described by Shervington *et al.*, (2009).

HSPA antigens detected using an Anti-mouse IgG FITC conjugated secondary antibody were identified, pre- and post-hypoxic treatment, in both the nucleus and the cytoplasm of the three glioma cell lines; 1321N1 (Figures 5.7 – 5.14), GOS-3 (Figures 5.15 – 5.20) and U87-MG (Figures 5.21 – 5.26). As with mRNA expression in the three glioma cell

lines, HSPA protein was detected pre-and post-hypoxia treatment in both the cytoplasm and the nucleus.

HSPA protein was detected in the cytoplasm in the normal astrocyte cell line, NHA with only limited fluorescence emitted from the nucleus of the cells. However, post-hypoxia, fluorescence was observed in both the cytoplasm and the nucleus (Figures 5.3 – 5.8). HSPA protein had migrated into the nucleus during hypoxia treatment, which correlates with the increased *HSPA* transcript levels, as shown in Figure 5.1.

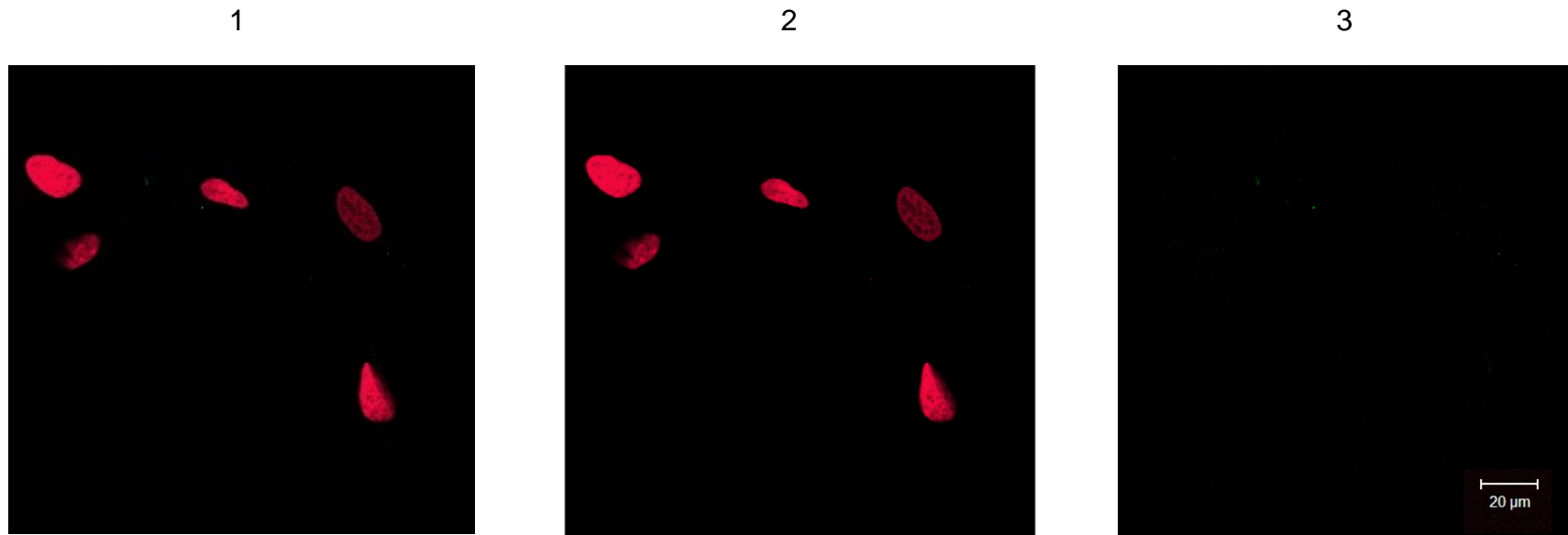


Figure 5.3 HSPA protein levels assessed using immunofluorescence for negative control of un-treated NHA cells. (1) Negative control whereby primary antibody HSPA has been omitted, Anti-mouse IgG FITC conjugated secondary antibody (green), no staining, nuclei labelled with propidium iodide (red); (2) nuclei staining labelled with propidium iodide (red) and (3) Anti-mouse IgG FITC conjugated secondary antibody (green), no staining. Objective = x 40 magnification. Scale bar = 20µm.

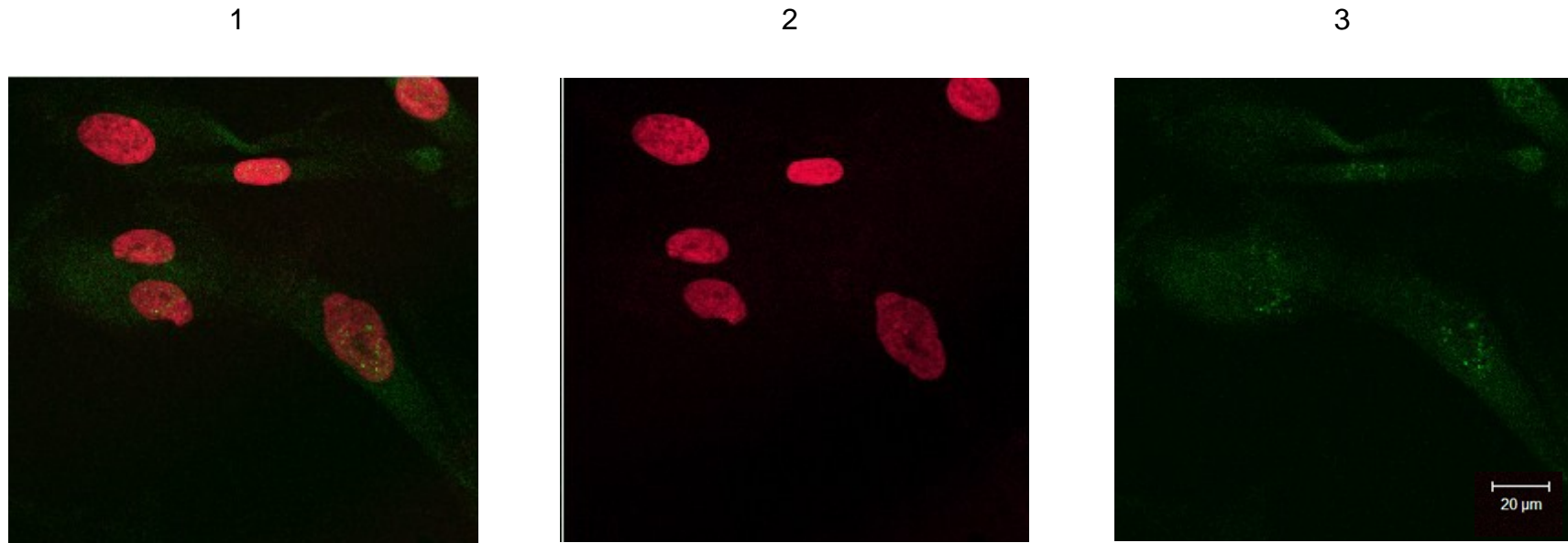


Figure 5.4 HSPA protein levels assessed using immunofluorescence in untreated NHA cells. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20µm.

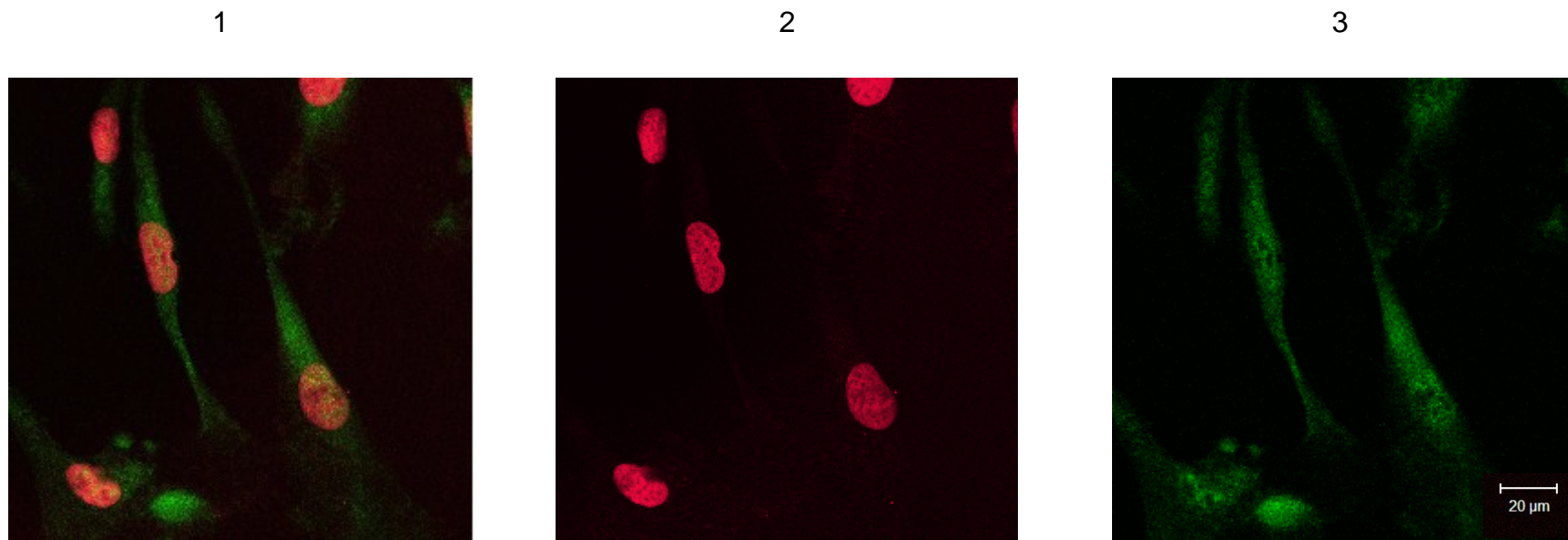


Figure 5.5 HSPA protein levels assessed using immunofluorescence in in post hypoxia treated NHA cells after 0 h recovery. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

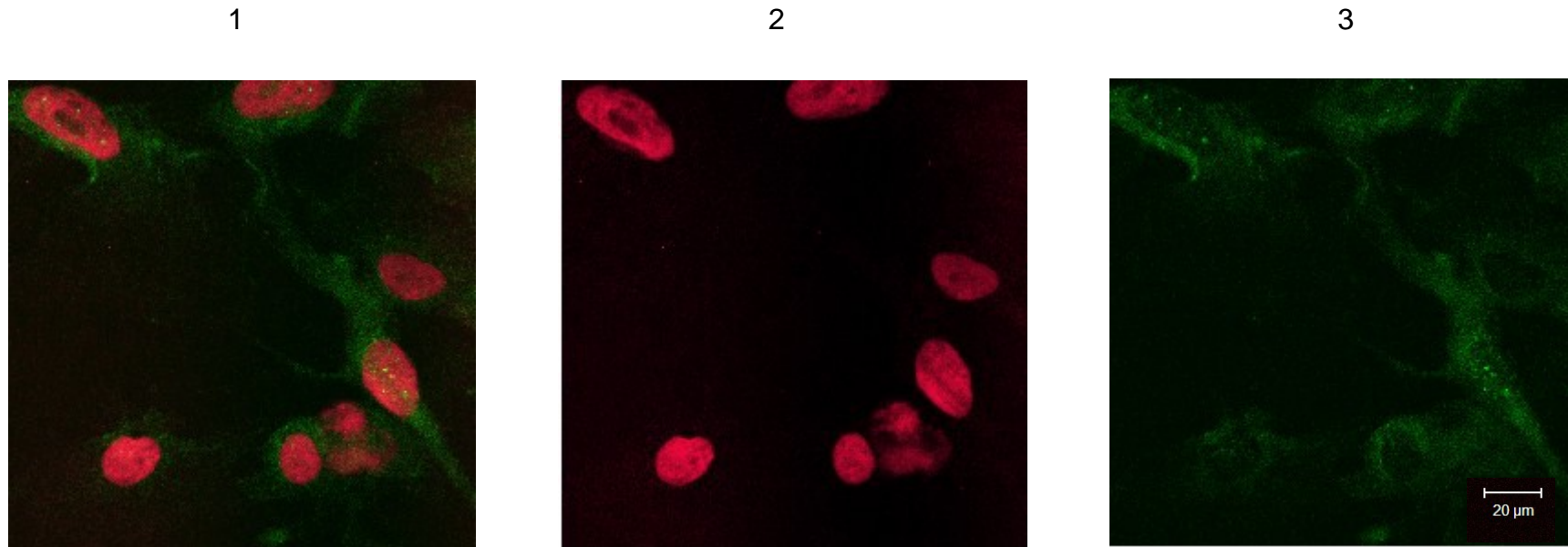


Figure 5.6 HSPA protein levels assessed using immunofluorescence in in post hypoxia treated NHA cells after 3 h recovery. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20µm.

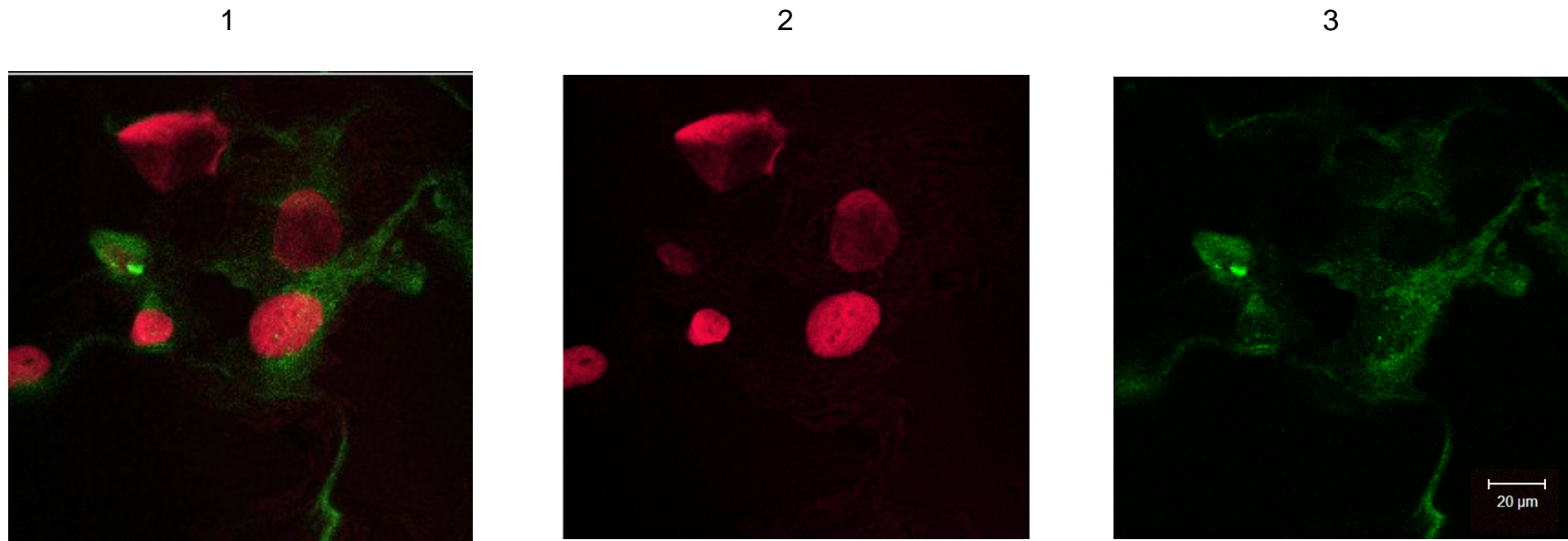


Figure 5.7 HSPA protein levels assessed using immunofluorescence in in post hypoxia treated NHA cells after 6 h recovery. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20µm.

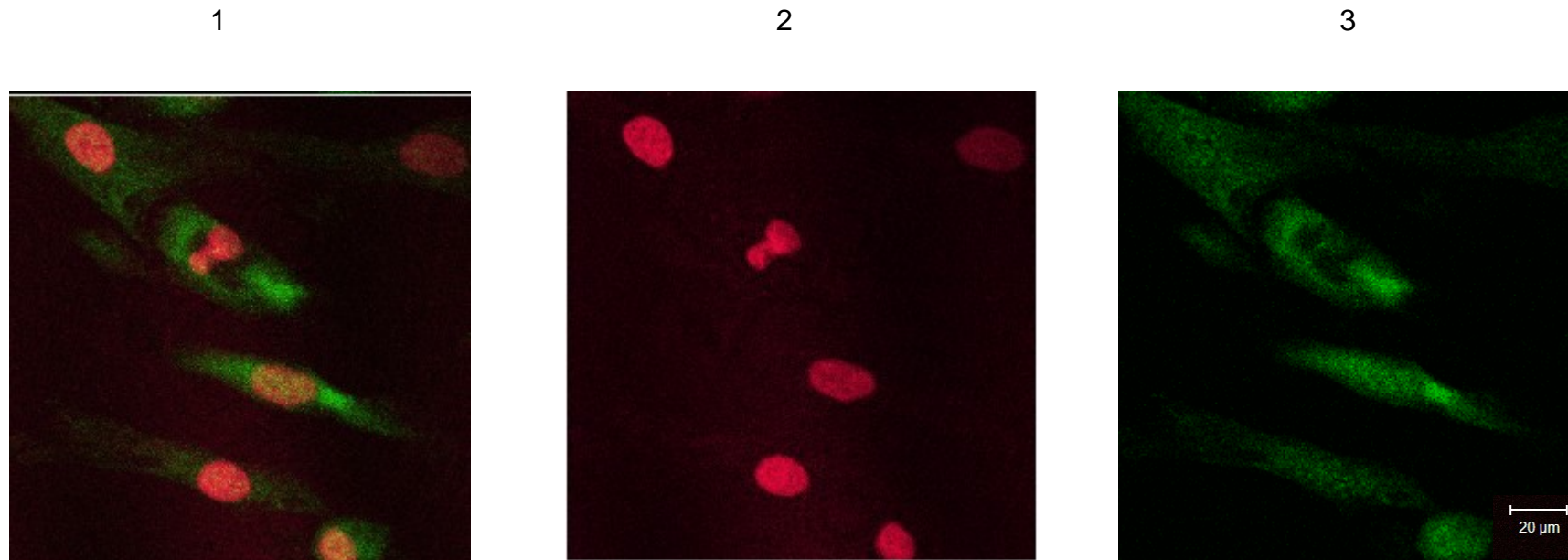


Figure 5.8 HSPA protein levels assessed using immunofluorescence in in post hypoxia treated NHA cells after 24 h recovery. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

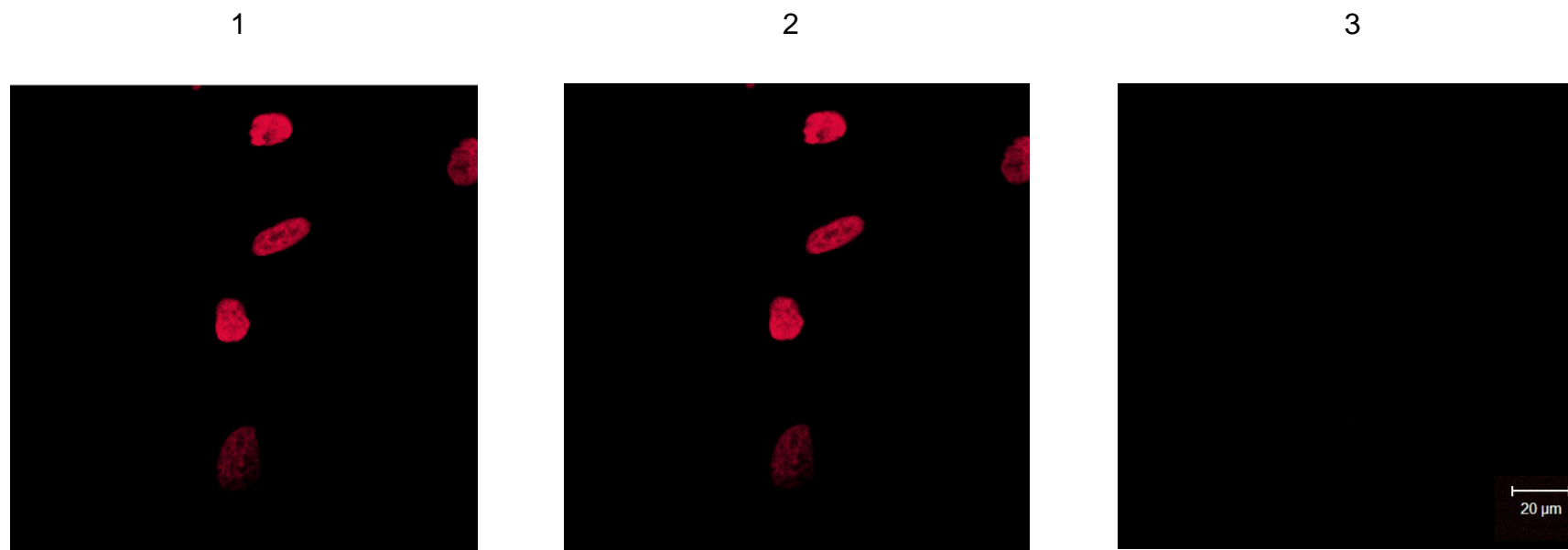


Figure 5.9 HSPA protein levels assessed using immunofluorescence for negative control of un-treated 1321N1 cells. (1) Negative control whereby primary antibody HSPA has been omitted, Anti-mouse IgG FITC conjugated secondary antibody (green), no staining, nuclei labelled with propidium iodide (red); (2) nuclei staining labelled with propidium iodide (red) and (3) Anti-mouse IgG FITC conjugated secondary antibody (green), no staining. Objective = x 40 magnification. Scale bar = 20µm.

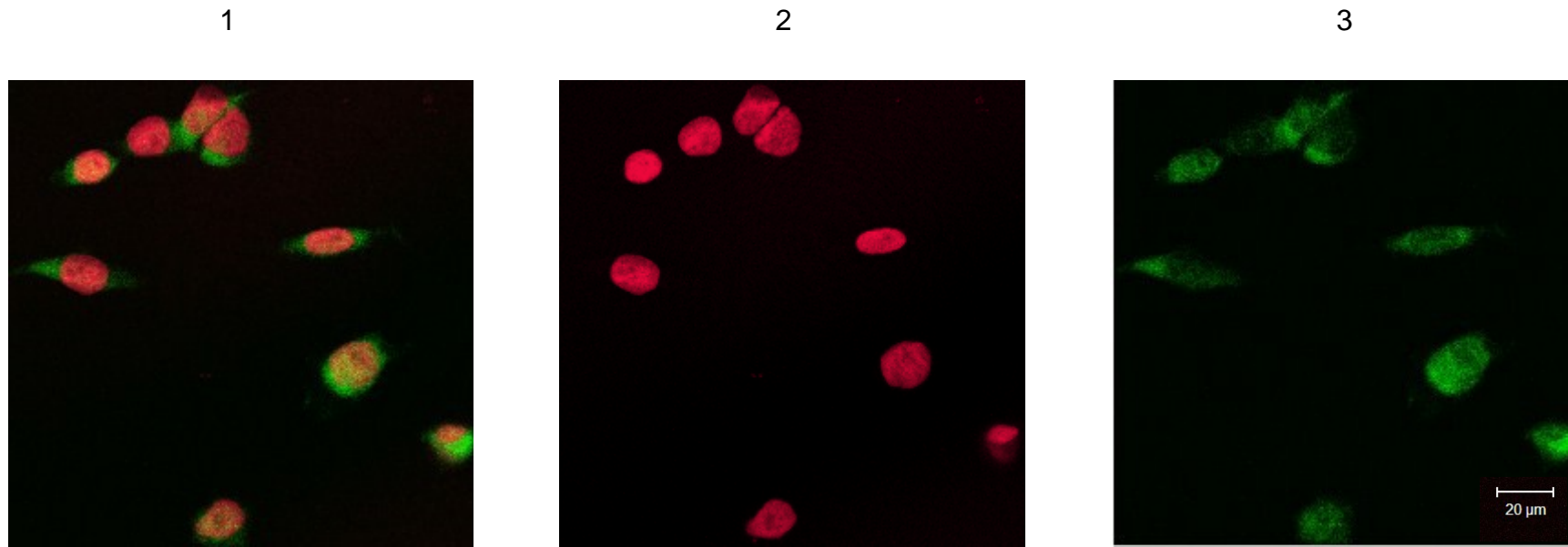


Figure 5.10 HSPA protein levels assessed using immunofluorescence in untreated 1321N1 cells. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

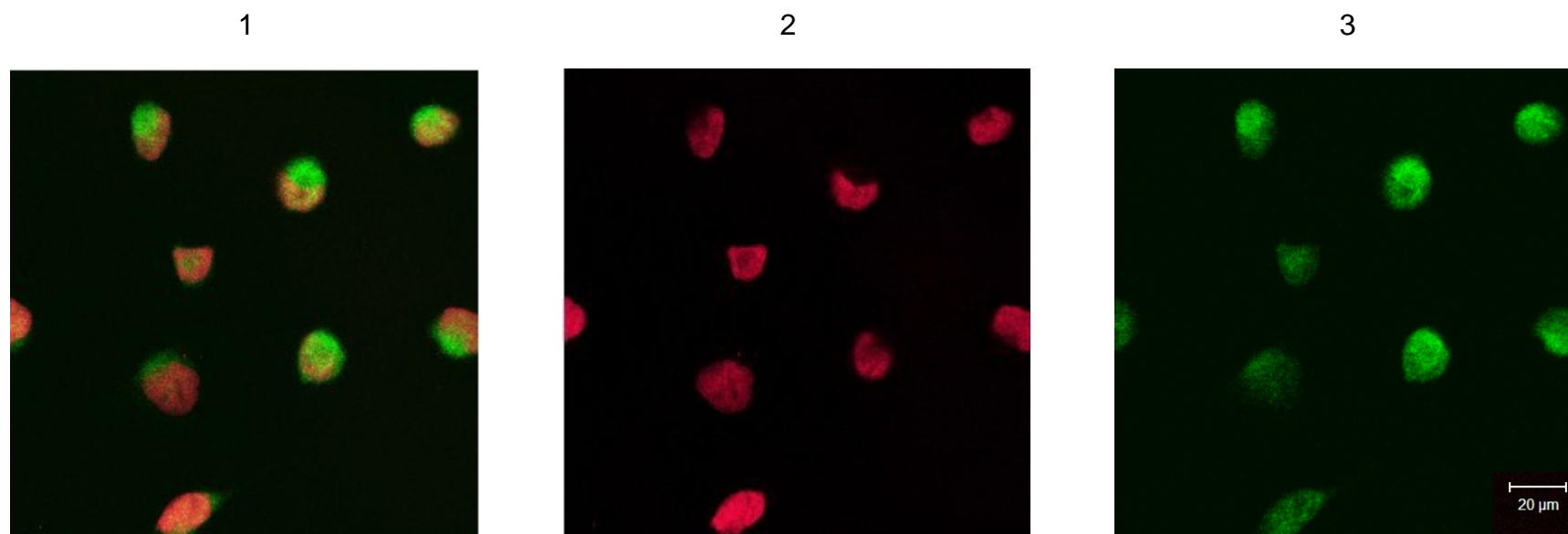


Figure 5.11 HSPA protein levels assessed using immunofluorescence in in post hypoxia treated 1321N1 cells after 0 h recovery. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

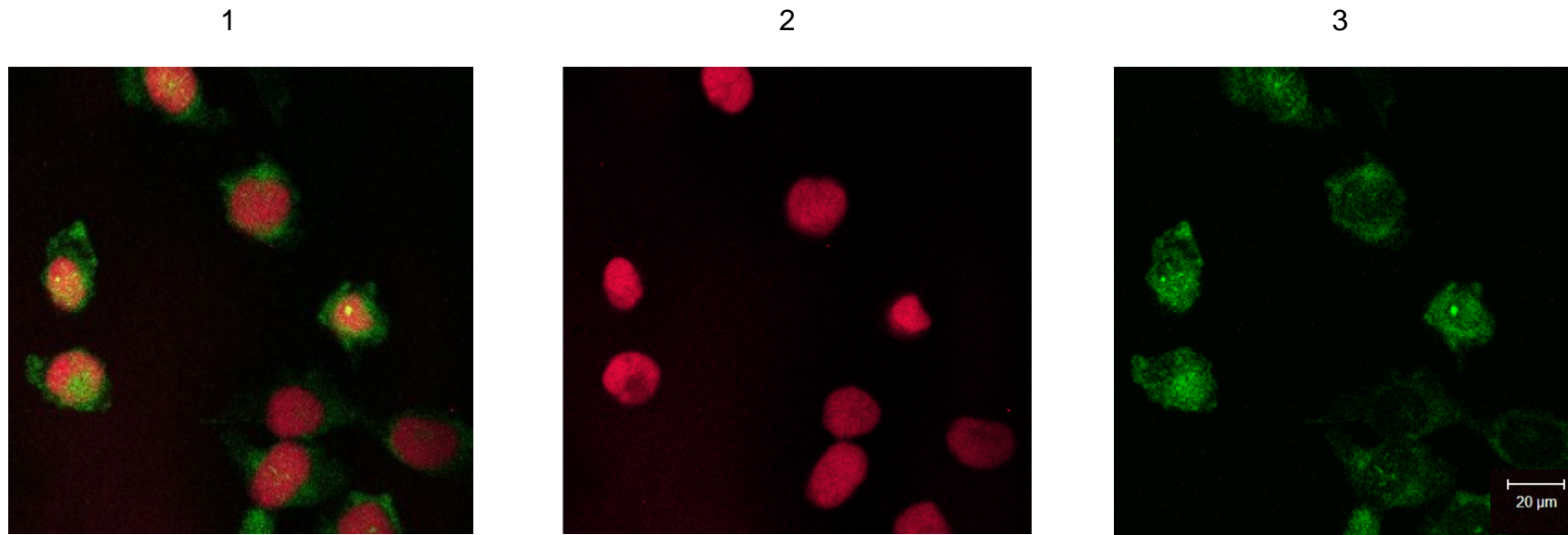


Figure 5.12 HSPA protein levels assessed using immunofluorescence in in post hypoxia treated 1321N1 cells after 3 h recovery. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

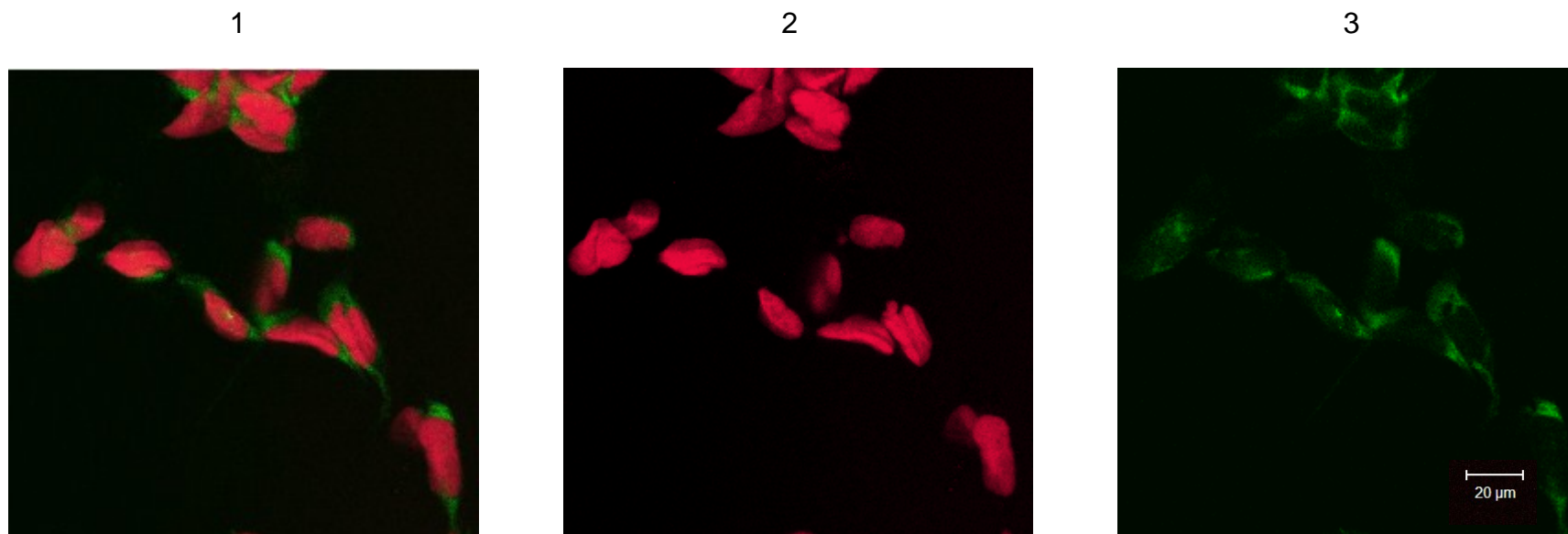


Figure 5.13 HSPA protein levels assessed using immunofluorescence in in post hypoxia treated 1321N1 cells after 6 h recovery. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

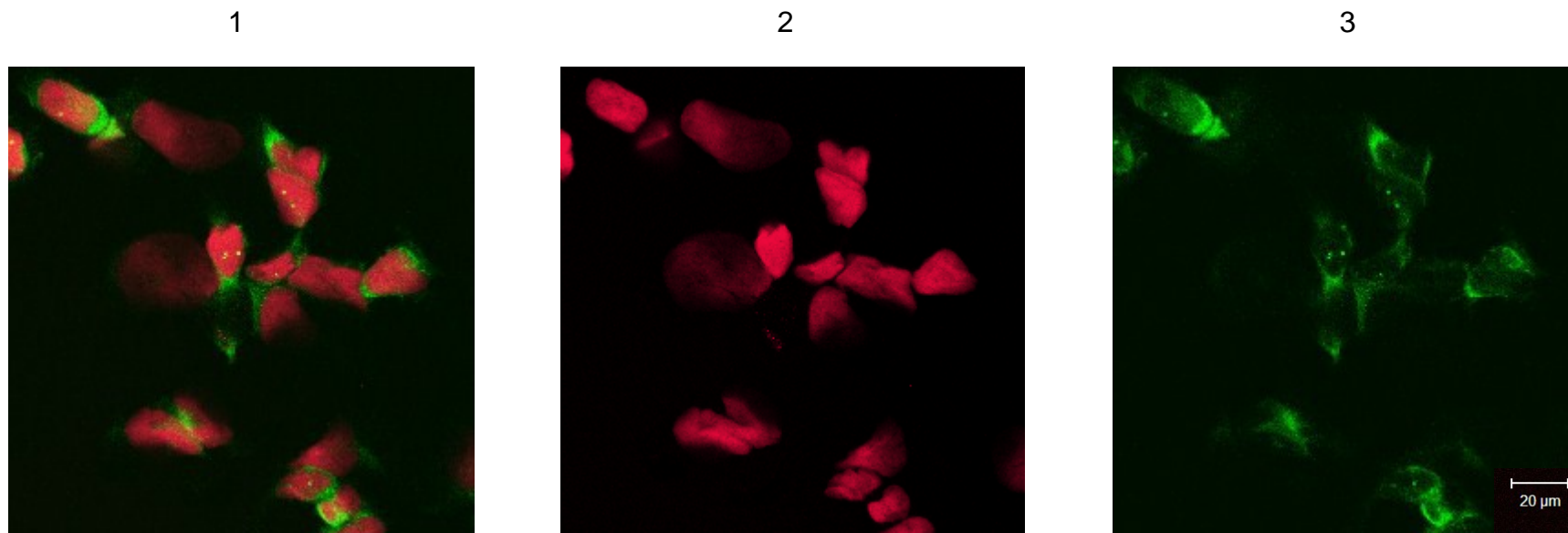


Figure 5.14 HSPA protein levels assessed using immunofluorescence in in post hypoxia treated 1321N1 cells after 24 h recovery. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20µm.

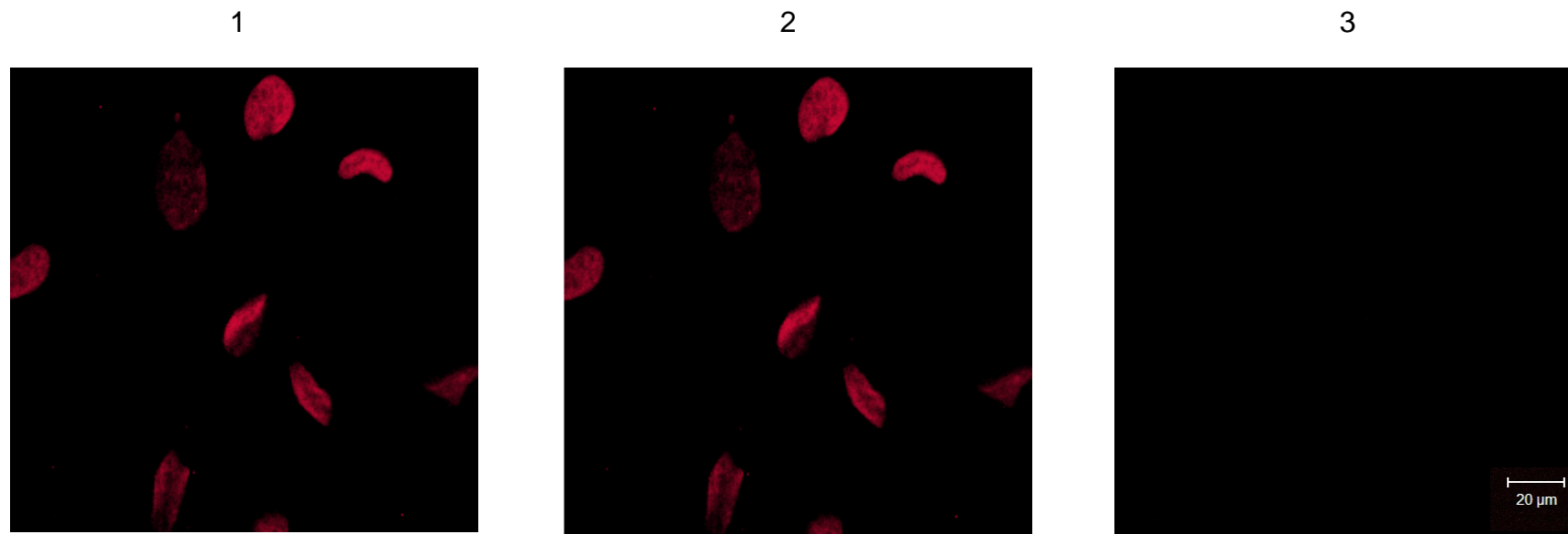


Figure 5.15 HSPA protein levels assessed using immunofluorescence for negative control of un-treated GOS-3 cells. (1) Negative control whereby primary antibody HSPA has been omitted, Anti-mouse IgG FITC conjugated secondary antibody (green), no staining, nuclei labelled with propidium iodide (red); (2) nuclei staining labelled with propidium iodide (red) and (3) Anti-mouse IgG FITC conjugated secondary antibody (green), no staining. Objective = x 40 magnification. Scale bar = 20μm.

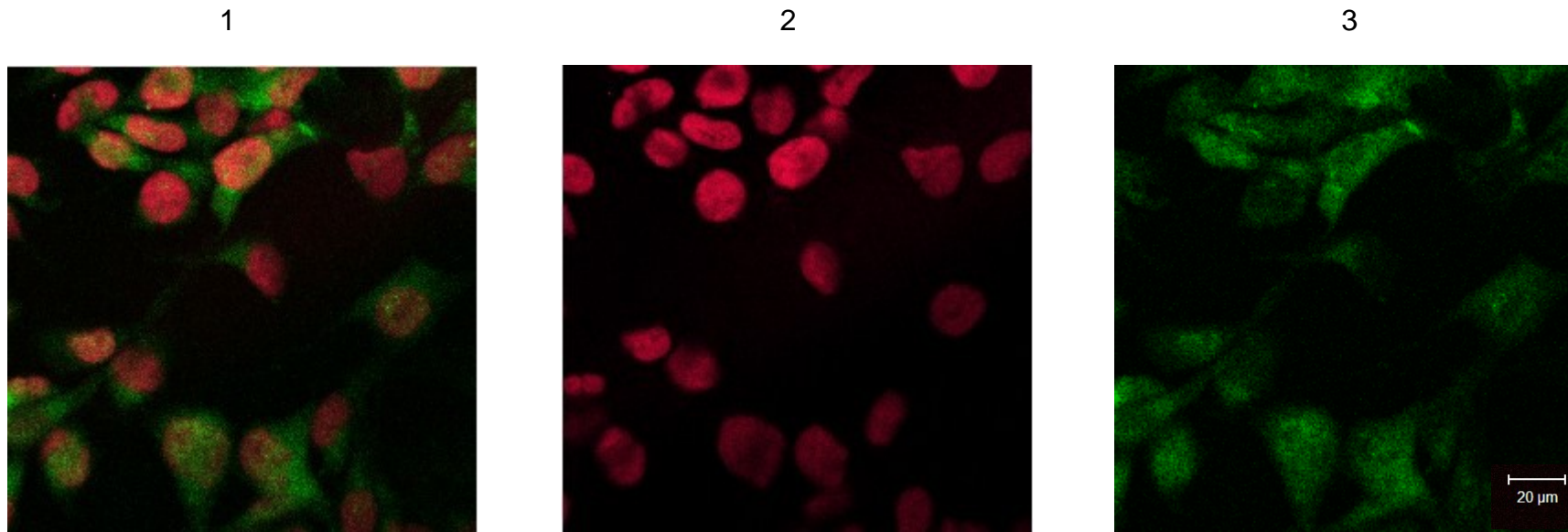


Figure 5.16 HSPA protein levels assessed using immunofluorescence in untreated GOS-3 cells. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

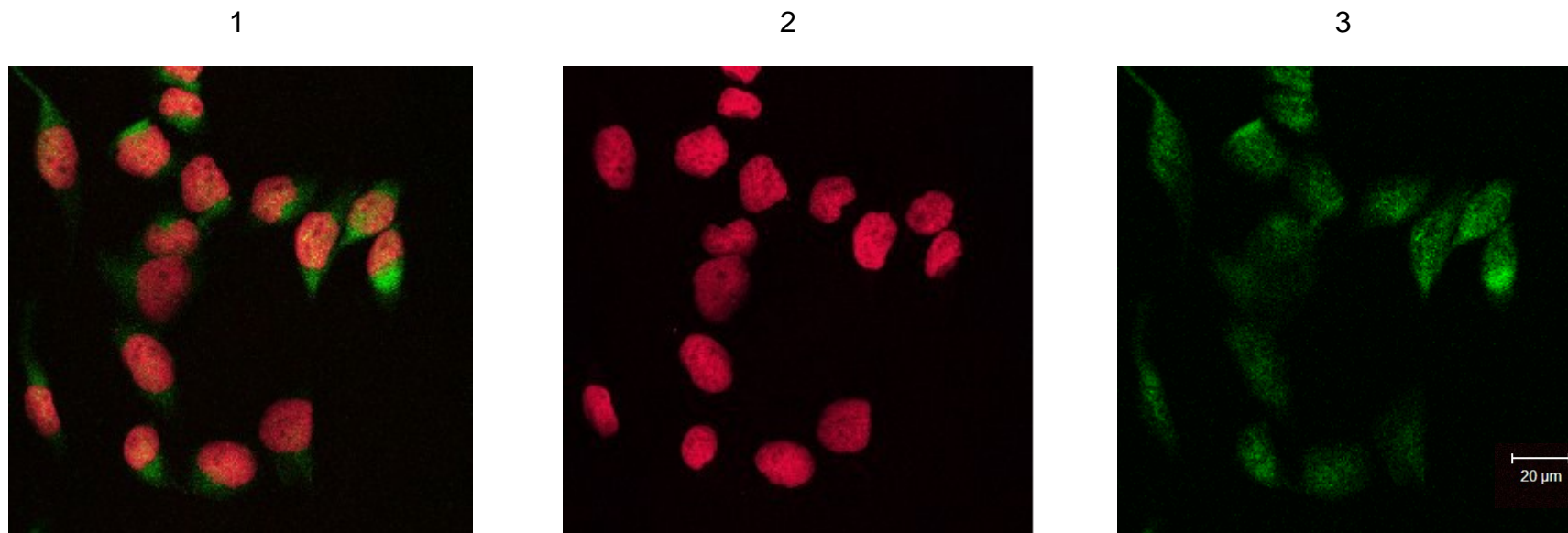


Figure 5.17 HSPA protein levels assessed using immunofluorescence in in post hypoxia treated GOS-3 cells after 0 h recovery. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20µm.

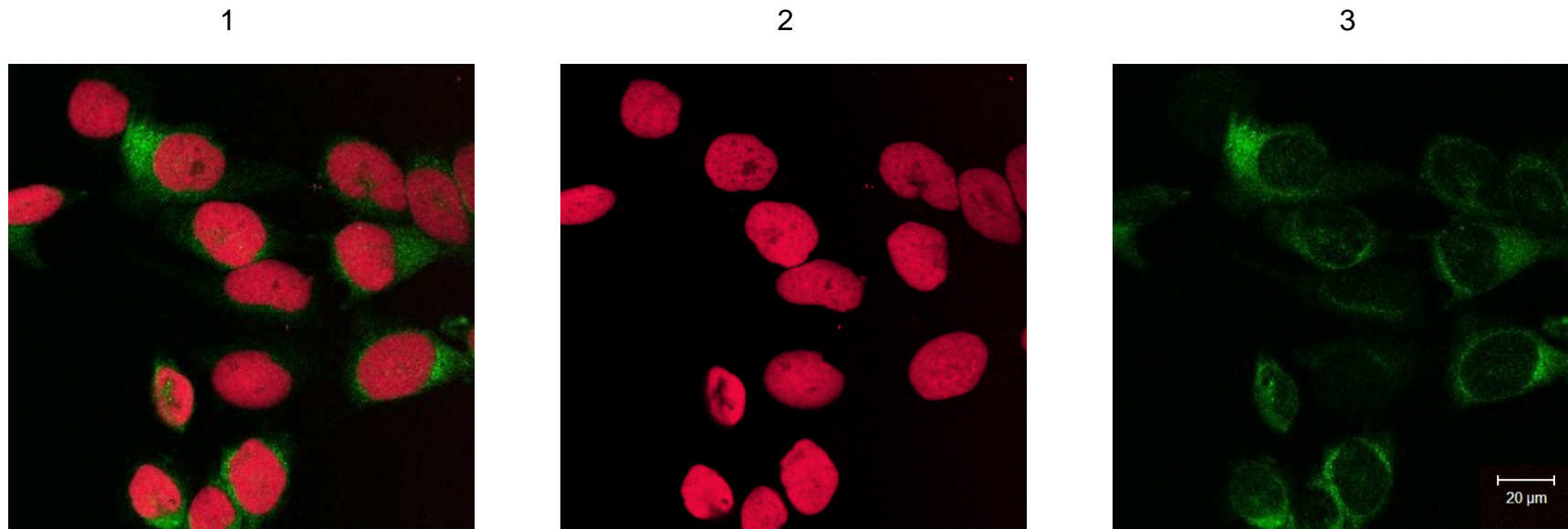


Figure 5.18 HSPA protein levels assessed using immunofluorescence in in post hypoxia treated GOS-3 cells after 3 h recovery. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20µm.

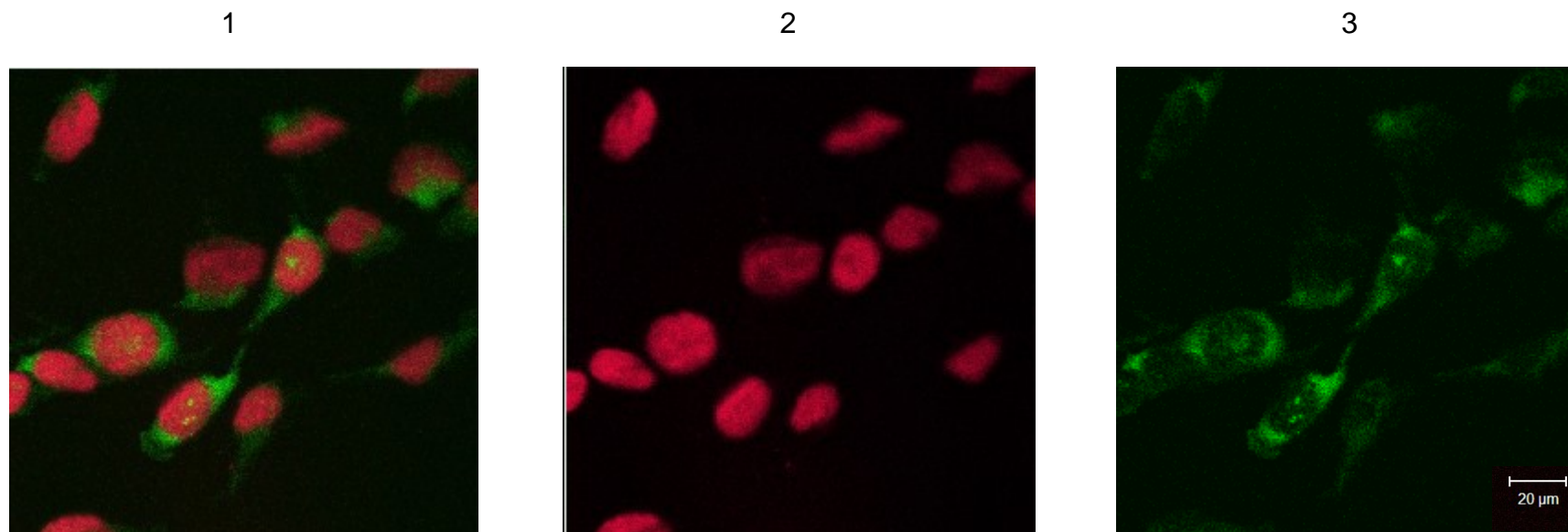


Figure 5.19 HSPA protein levels assessed using immunofluorescence in in post hypoxia treated GOS-3 cells after 6 h recovery. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20µm.

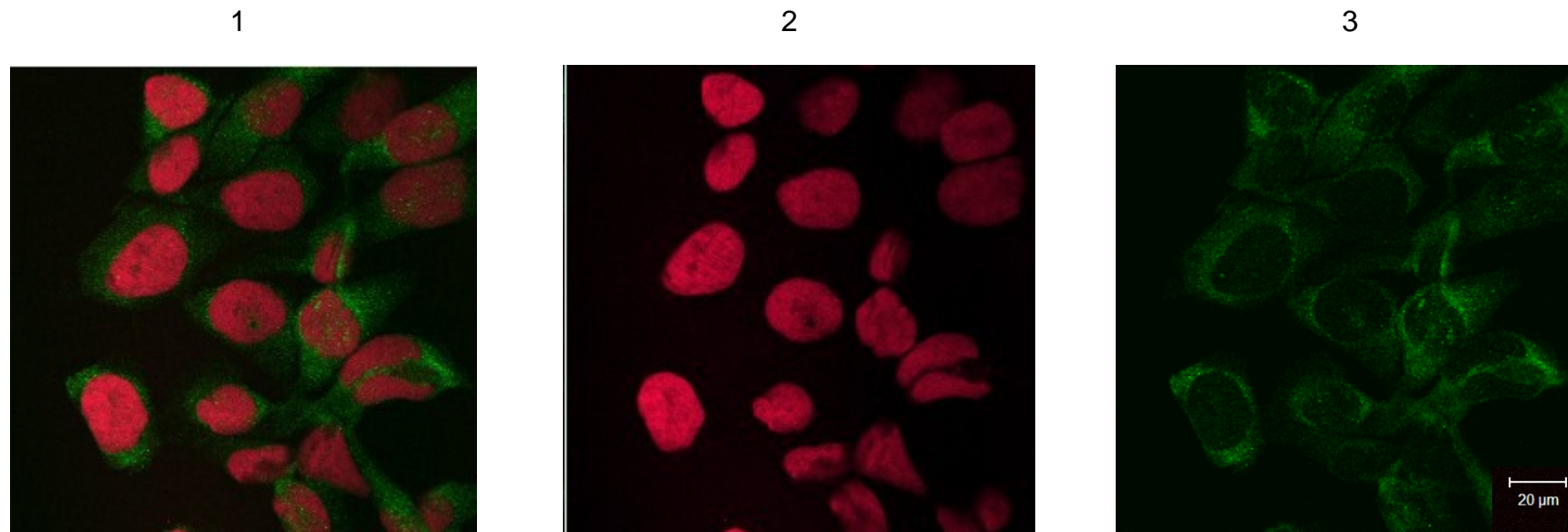


Figure 5.20 HSPA protein levels assessed using immunofluorescence in in post hypoxia treated GOS-3 cells after 24 h recovery. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20µm.

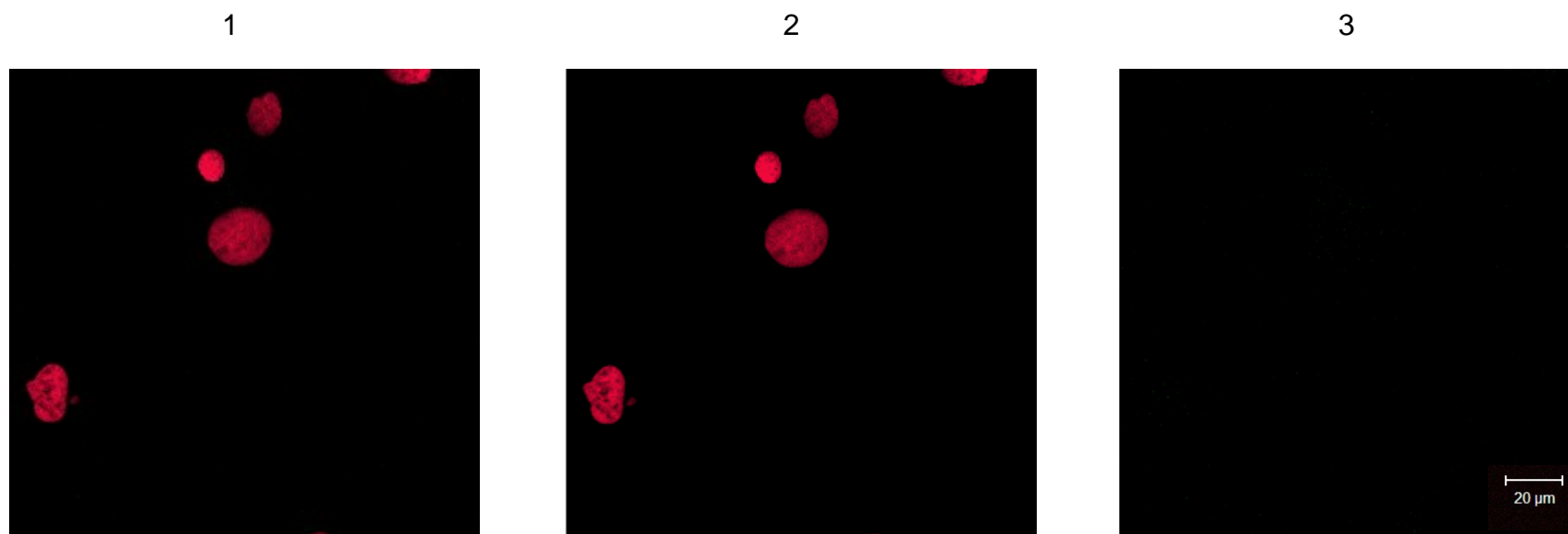


Figure 5.21 HSPA protein levels assessed using immunofluorescence for negative control of un-treated U87-MG cells. (1) Negative control whereby primary antibody HSPA has been omitted, Anti-mouse IgG FITC conjugated secondary antibody (green), no staining, nuclei labelled with propidium iodide (red); (2) nuclei staining labelled with propidium iodide (red) and (3) Anti-mouse IgG FITC conjugated secondary antibody (green), no staining. Objective = x 40 magnification. Scale bar = 20µm.

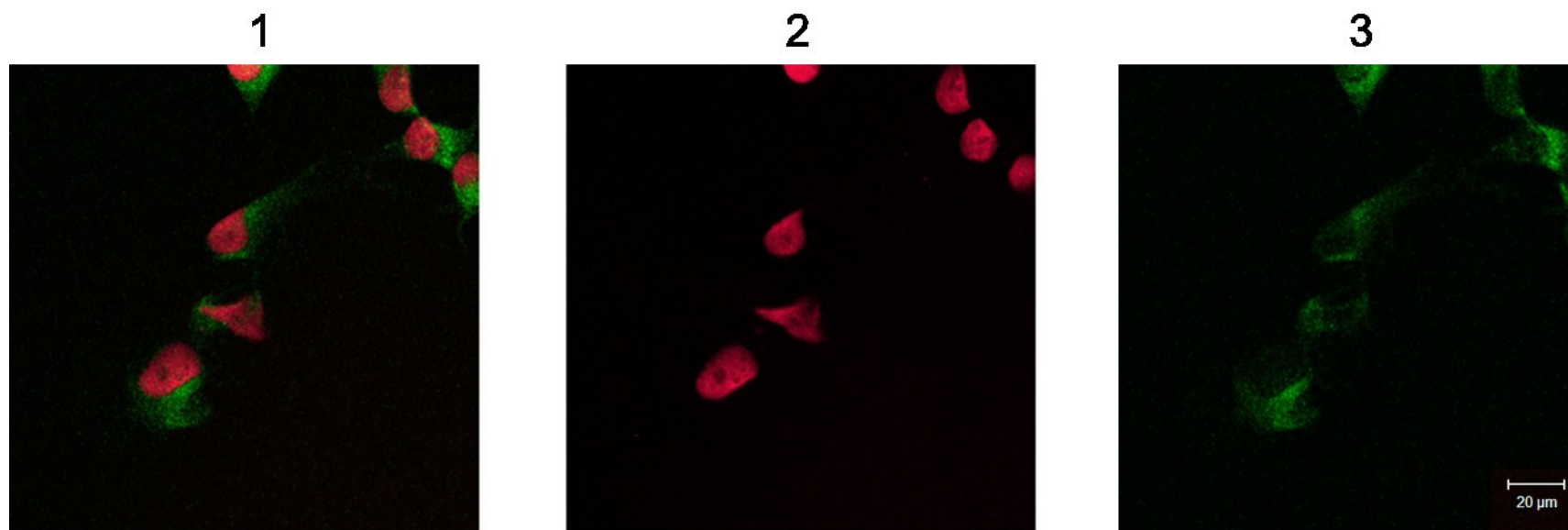


Figure 5.22 HSPA protein levels assessed using immunofluorescence in untreated U87-MG cells. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20µm.

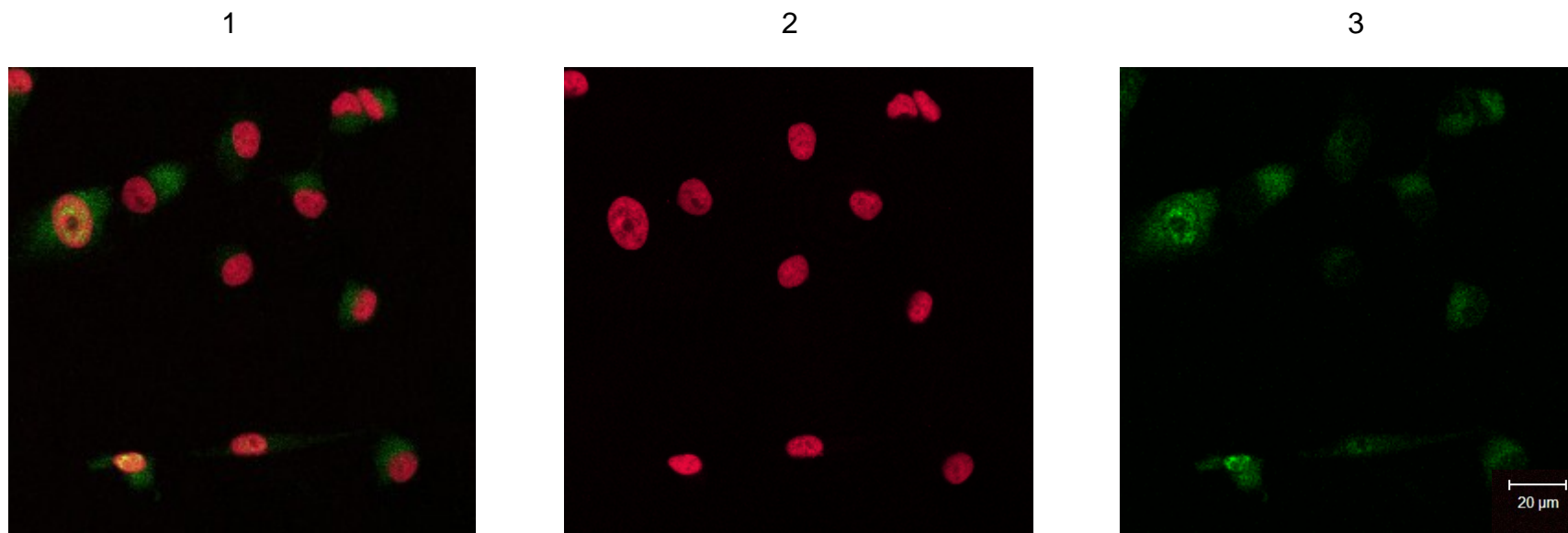


Figure 5.23 HSPA protein levels assessed using immunofluorescence in in post hypoxia treated U87-MG cells after 0 h recovery. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20µm.

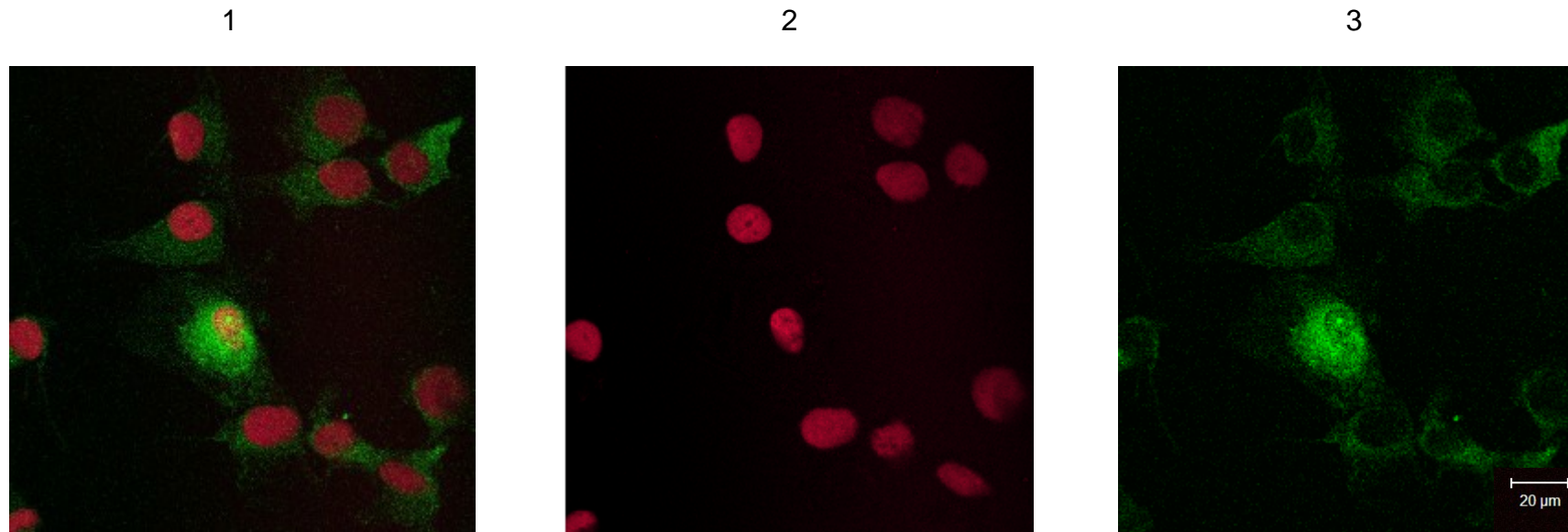


Figure 5.24 HSPA protein levels assessed using immunofluorescence in in post hypoxia treated U87-MG cells after 3 h recovery. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

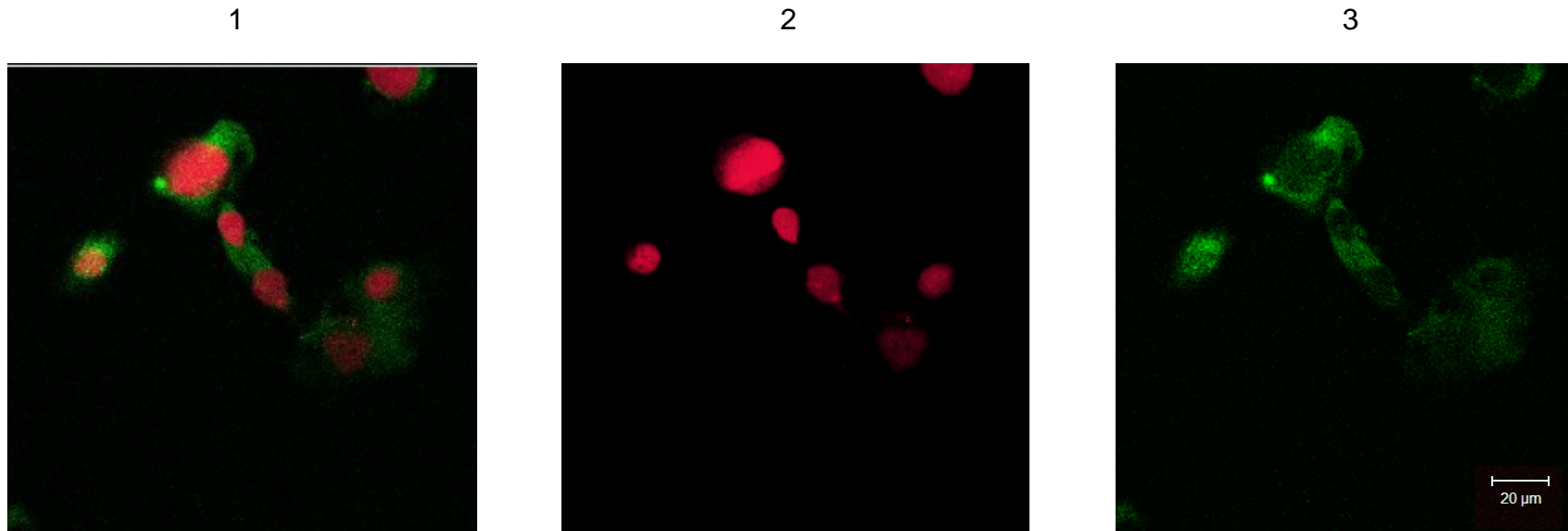


Figure 5.25 HSPA protein levels assessed using immunofluorescence in in post hypoxia treated U87-MG cells after 6 h recovery. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

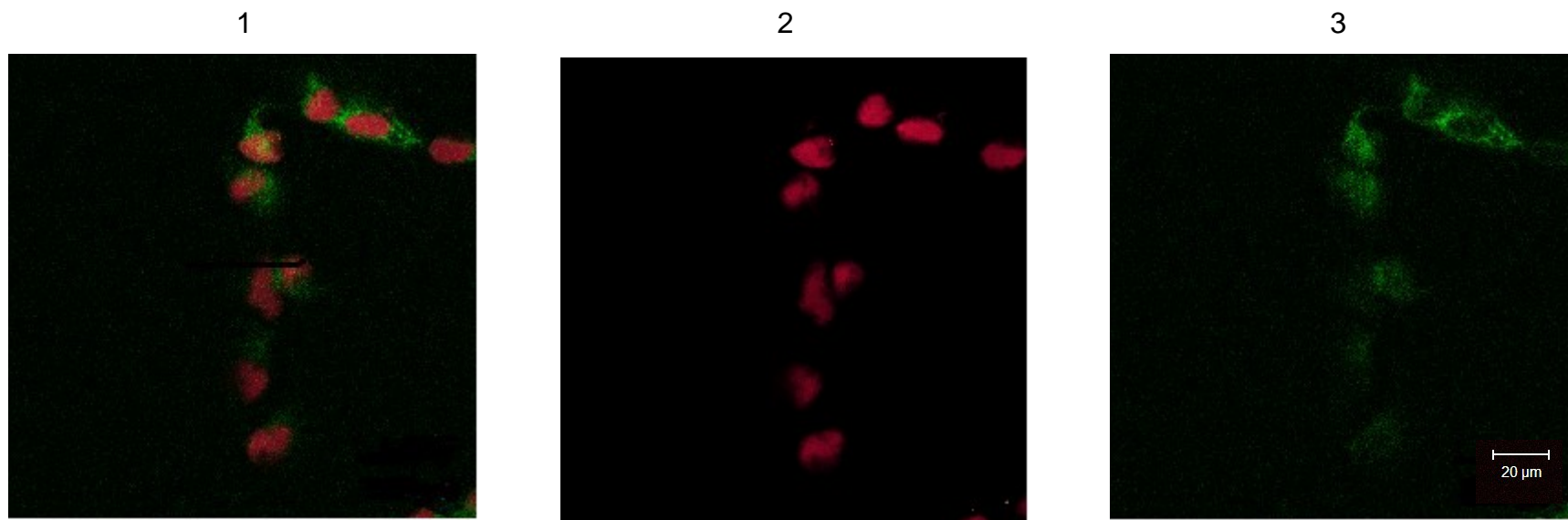


Figure 5.26 HSPA protein levels assessed using immunofluorescence in in post hypoxia treated U87-MG cells after 24 h recovery. (1) combined nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green); (2) nuclei staining labelled with propidium iodide (red) and (3) primary antibody HSPA detected with Anti-mouse IgG FITC conjugated antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

5.4 Flow Cytometry

To accurately quantify production of the protein HSPA in cells, flow cytometry analysis was carried out using the same four cell lines for pre- and post-hypoxia conditions as in previous sections of this chapter. Cultured cells were stained and subjected to flow cytometry, briefly 1×10^6 were washed once in 0.1% BSA in PBS before being re-suspended in 0.1% Triton X-100 for 15 min. Following incubation cells were collected and washed once with 0.1% BSA in PBS before being blocked in 5% goat serum in 0.1% BSA in PBS for 30 min. HSPA primary antibody (1:200) diluted in blocking solution was applied for 30 min. Cells were then washed with 0.1% BSA in PBS and light sensitive Anti-Mouse IgG FITC conjugated secondary antibody (1:200) diluted in blocking solution was added for 30 min. Following incubation cells were washed twice in 0.1% BSA in PBS before finally being re-suspended in an aliquot 0.1% BSA in PBS and filtered into FACS tubes. All incubation steps were performed on ice.

This analysis showed a higher relative fluorescence intensity in hypoxic 1321N1, GOS-3 and U87-MG cell lines (approximately 3-fold) compared to NHA, reflecting a higher level of production of HSPA protein (Figures 5.28 – 5.32, NHA, 5.33 – 5.37, 1321N1, 5.38 – 5.42, GOS-3, 5.43 – 5.47, U87-MG). In pre and post hypoxia treated 1321N1, GOS-3 and U87-MG glioma cells, HSPA protein levels were relatively high, with approximately 97%, 89% and 97% respectively showing positive for HSPA in pre hypoxia treatment, with approximately 99%, 73% and 84% respectively showing positive for HSPA in post hypoxia treatment after 0 h recovery, with approximately 99%, 92% and 91% respectively showing positive for HSPA in post hypoxia treatment after 3 h recovery with approximately 99%, 92% and 96% respectively showing positive for HSPA in post hypoxia treatment after 6 h recovery, and approximately

94%, 97% and 96% respectively showing positive for HSPA in post hypoxia treatment after 24 h recovery compared to the NHA cells showing positive for HSPA pre hypoxia treatment approximately 0.10% and post hypoxia treatment after 0 h recovery, approximately 33%; after 3 h recovery approximately 31%; after 6 h recovery approximately 25% and after 24 h recovery approximately 11% (Figure 5.27 and Table 5.3).

In GOS-3 and U87-MG glioma cells, hypoxic treatment resulted in a transient decrease in HSPA protein at 0 h recovery period from, approximate percentage level: 89 to 73 for GOS-3 and from 97 to 84 for U87-MG. After a three hour recovery period, HSPA protein levels increased to pre-hypoxic levels and remained relatively constant up to 24 h. HSPA protein synthesis in the NHA cell line reached maximal levels of approximately 33% at the 0 h recovery period after hypoxia treatment, followed by a progressive decrease in HSPA protein levels up to the 24 h recovery period (Figure 5.27). A shift of the HSPA detection peak was observed from 1×10^2 to 1×10^4 in the NHA cell line (Figures 5.28 – 5.32), however, no such shift was observed in 1321N1, GOS-3 and U87-MG glioma cell lines.

A

| Cell Line | Pre-hypoxia | Post-hypoxia (Hours) | | | |
|-----------|-------------|----------------------|------|------|------|
| | | 0 | 3 | 6 | 24 |
| NHA | 0 | 33.0 | 30.8 | 24.7 | 11.3 |
| 1321N1 | 97.1 | 99.6 | 99.1 | 99.0 | 93.6 |
| GOS-3 | 89.2 | 73.4 | 91.9 | 92.0 | 96.6 |
| U87-MG | 96.9 | 83.7 | 90.9 | 96.5 | 96.3 |

B

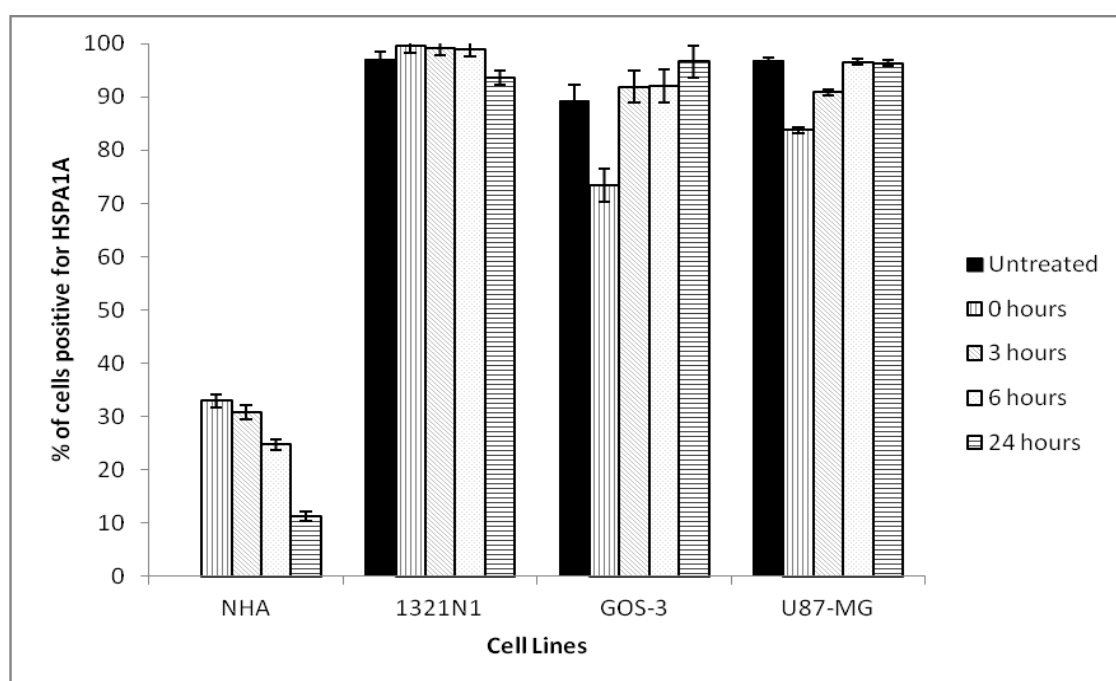


Figure 5.27 Average percentage of cells showing positive for HSPA in pre and post hypoxia treatment. (A) Average percentage of normal and glioma cells positive for HSPA pre and post hypoxia detected by flow cytometry, considering 10,000 events per sample. (B) Histogram showing the mean percentage of cells evaluated by flow cytometry of three independent experiments, and the standard deviation from this mean is shown by the error bars. No column was shown on the histogram for NHA as the value = 0.

5.4.1 Statistical Analysis

A two-way factorial mixed 4 (cell line: NHA, 1321N1, GOS-3, U87-MG) x 5 (treatment: untreated, 0, 3, 6 and 24 h recovery after hypoxia treatment) ANOVA analysis of variance test was performed to investigate differences of HSPA protein levels. The means and standard deviation are presented in Table 5.3.

Table 5.3. Descriptive statistics showing the mean HSPA protein levels for untreated and treated cell lines.

| Treatment | Cell Line | N | Mean HSPA protein level | STD. Deviation |
|---------------------------------------|-----------|---|-------------------------|----------------|
| Pre-hypoxia | NHA | 3 | .13 | .058 |
| | 1321N1 | 3 | 96.87 | .322 |
| | GOS-3 | 3 | 89.15 | .050 |
| | U87-MG | 3 | 96.85 | .050 |
| 0 h recovery after hypoxia treatment | NHA | 3 | 32.50 | .500 |
| | 1321N1 | 3 | 99.55 | .050 |
| | GOS-3 | 3 | 73.38 | .076 |
| | U87-MG | 3 | 83.6 | .100 |
| 3 h recovery after hypoxia treatment | NHA | 3 | 30.83 | .153 |
| | 1321N1 | 3 | 99.05 | .050 |
| | GOS-3 | 3 | 91.85 | .050 |
| | U87-MG | 3 | 90.85 | .050 |
| 6 h recovery after hypoxia treatment | NHA | 3 | 24.72 | .126 |
| | 1321N1 | 3 | 99.03 | .061 |
| | GOS-3 | 3 | 91.83 | .764 |
| | U87-MG | 3 | 96.45 | .050 |
| 24 h recovery after hypoxia treatment | NHA | 3 | 11.30 | .095 |
| | 1321N1 | 3 | 93.53 | .060 |
| | GOS-3 | 3 | 96.62 | .076 |
| | U87-MG | 3 | 96.33 | .058 |

Overall inspection of the mean scores for HSPA protein levels indicated that untreated and treated 1321N1; GOS-3 and U87-MG cell lines were considerably higher than the NHA cell line (Table 5.3).

There was a significant effect for: cell line [$F(15,11.44) = 32736$; $P < 0.001$]; Wilks' Lambda = 0.000, partial eta squared = 1.000; a significant effect for treatment [$F(4,5) = 6262.1$; $P < 0.001$], Wilks' Lambda = 0.000, partial eta squared = 1.000. There was a significant effect in the interaction between treatment and cell line, [$F(12,13.52) = 5234.5$; $P < 0.001$], Wilks' Lambda = 0.000, partial eta squared = 0.999.

There was a statistically significant main effect for cell lines on the combined variables: [$F(3,8) = 406583$, $P < .001$]; partial eta squared (η_p^2) = 1.0.

When the results for the dependent variables were considered separately, there was a statistically high significance in untreated [$F(3,8) = 239590$, $P < .000$]; partial eta squared (η_p^2) = 1.000. Post-hoc analyses using Tukey's HSD test indicated significantly lower HSPA protein levels in untreated NHA than in: 1321N1 (MD = 96.73, $P < .05$); GOS-3 (MD = 89.02, $P < .05$) and U87-MG (MD = 96.72, $P < .05$). There was significantly lower HSPA protein levels in untreated 1321N1 than in GOS-3 (MD = 7.72, $P < .05$). There was significantly lower HSPA protein levels in untreated GOS-3 than in U87-MG (MD = 7.70, $P < .05$). All other differences between groups failed to reach significance (Table 5.4).

There was a statistically high significance after 0 h recovery [$F(3,8) = 36603$, $P < .000$]; partial eta squared (η_p^2) = 1.000. Post-hoc analyses using Tukey's HSD test indicated significantly lower HSPA protein levels after 0 h recovery in NHA than in: 1321N1 (MD = 67.05, $P < .05$); GOS-3 (MD = 40.88, $P < .05$) and U87-MG (MD = 51.10, $P < .05$). There were significantly lower HSPA protein levels after 0 h recovery in 1321N1 than in: GOS-3 (MD = 7.72, $P < .05$) and U87-MG (MD = 15.95, $P < .05$). There was significantly lower HSPA protein levels after 0 h recovery in GOS-3 than in U87-MG (MD = 10.22, $P < .05$) (Table 5.4).

There was a statistically high significance after 3 h recovery [$F(3,8) = 392388$, $P < .000$]; partial eta squared (η_p^2) = 1.000. Post-hoc analyses using Tukey's HSD test indicated significantly lower HSPA protein levels after 3 h recovery in NHA than in: 1321N1 (MD = 68.22, $P < .05$); GOS-3 (MD = 61.02, $P < .05$) and U87-MG (MD = 60.02, $P < .05$). There were significantly lower HSPA protein levels after 3 h recovery in 1321N1 than in: GOS-3 (MD = 7.20, $P < .05$) and U87-MG (MD = 8.20, $P < .05$). There was significantly lower HSPA protein levels after 3 h recovery in GOS-3 than in U87-MG (MD = 1.00, $P < .05$) (Table 5.4).

There was a statistically high significance after 6 h recovery [$F(3,8) = 25196$, $P < .000$]; partial eta squared (η_p^2) = 1.000. Post-hoc analyses using Tukey's HSD test indicated significantly lower HSPA protein levels after 6 h recovery in NHA than in: 1321N1 (MD = 74.31, $P < .05$); GOS-3 (MD = 67.12, $P < .05$) and U87-MG (MD = 71.73, $P < .05$). There were significantly lower HSPA protein levels after 6 h recovery in 1321N1 than in: GOS-3 (MD = 7.20, $P < .05$) and U87-MG (MD = 2.58, $P < .05$). There was significantly lower HSPA protein levels after 6 h recovery in GOS-3 than in U87-MG (MD = 4.62, $P < .05$) (Table 5.4).

There was a statistically high significance after 24 h recovery [$F(3,8) = 972016$, $P < .000$]; partial eta squared (η_p^2) = 1.000. Post-hoc analyses using Tukey's HSD test indicated significantly lower HSPA protein levels after 24 h recovery in NHA than in: 1321N1 (MD = 82.23, $P < .05$); GOS-3 (MD = 85.31, $P < .05$) and U87-MG (MD = 85.03, $P < .05$). There were significantly lower HSPA protein levels after 24 h recovery in 1321N1 than in: GOS-3 (MD = 3.09, $P < .05$) and U87-MG (MD = 2.80, $P < .05$). There was significantly lower HSPA protein levels after 24 h recovery in GOS-3 than in U87-MG (MD = .283, $P < .05$) (Table 5.4).

Table 5.4. Statistical data from Tukey's HSD test showing significance of HSPA protein levels in treated and untreated cell lines. Any differences that are not significant are highlighted in bold. Significance ≤ 0.05 .

| | | NHA | | 1321N1 | | GOS-3 | | U87-MG | |
|--------------|-----------|------------------------------|------|------------------------------------|-------------|------------------------------|------|------------------------------------|-------------|
| Treatment | Cell Line | Mean Diff \pm STD error | SIG. | Mean Diff \pm STD error | SIG. | Mean Diff \pm STD error | SIG. | Mean Diff \pm STD error | SIG. |
| Untreated | NHA | | | 96.73 \pm .136 | .000 | 89.02 \pm .136 | .000 | 96.72 \pm .136 | .000 |
| | 1321N1 | 96.73 \pm .136 | .000 | | | 7.72 \pm .136 | .000 | 0.017 \pm .136 | .999 |
| | GOS-3 | 89.02 \pm .136 | .000 | 7.72 \pm .136 | .000 | | | 7.70 \pm .136 | .000 |
| | U87-MG | 96.72 \pm .136 | .000 | 0.017 \pm .136 | .999 | 7.70 \pm .136 | .000 | | |
| 0h recovery | NHA | | | 67.05 \pm .212 | .000 | 40.88 \pm .212 | .000 | 51.1 \pm .212 | .000 |
| | 1321N1 | 67.05 \pm .212 | .000 | | | 26.17 \pm .212 | .000 | 15.95 \pm .212 | .000 |
| | GOS-3 | 40.88 \pm .212 | .000 | 26.17 \pm .212 | .000 | | | 10.22 \pm .212 | .000 |
| | U87-MG | 51.1 \pm .212 | .000 | 15.95 \pm .212 | .000 | 10.22 \pm .212 | .000 | | |
| 3h recovery | NHA | | | 68.21 \pm .072 | .000 | 61.02 \pm .072 | .000 | 60.02 \pm .072 | .000 |
| | 1321N1 | 68.21 \pm .072 | .000 | | | 7.20 \pm .072 | .000 | 8.20 \pm .072 | .000 |
| | GOS-3 | 61.02 \pm .072 | .000 | 7.20 \pm .072 | .000 | | | 1.0 \pm .072 | .000 |
| | U87-MG | 60.02 \pm .072 | .000 | 8.20 \pm .072 | .000 | 2.0 \pm .072 | .000 | | |
| 6h recovery | NHA | | | 74.31 \pm .318 | .000 | 67.12 \pm .318 | .000 | 71.73 \pm .318 | .000 |
| | 1321N1 | 74.31 \pm .318 | .000 | | | 7.20 \pm .318 | .000 | 2.58 \pm .318 | .000 |
| | GOS-3 | 67.12 \pm .318 | .000 | 7.20 \pm .318 | .000 | | | 4.62 \pm .318 | .000 |
| | U87-MG | 71.73 \pm .318 | .000 | 2.58 \pm .318 | .000 | 4.62 \pm .318 | .000 | | |
| 12h recovery | NHA | | | 82.23 \pm .060 | .000 | 85.31 \pm .060 | .000 | 85.03 \pm .060 | .000 |
| | 1321N1 | 82.23 \pm .060 | .000 | | | 3.09 \pm .060 | .000 | 2.80 \pm .060 | .000 |
| | GOS-3 | 85.31 \pm .060 | .000 | 3.09 \pm .060 | .000 | | | .283 \pm .060 | .007 |
| | U87-MG | 85.03 \pm .060 | .000 | 2.80 \pm .060 | .000 | .283 \pm .060 | .007 | | |

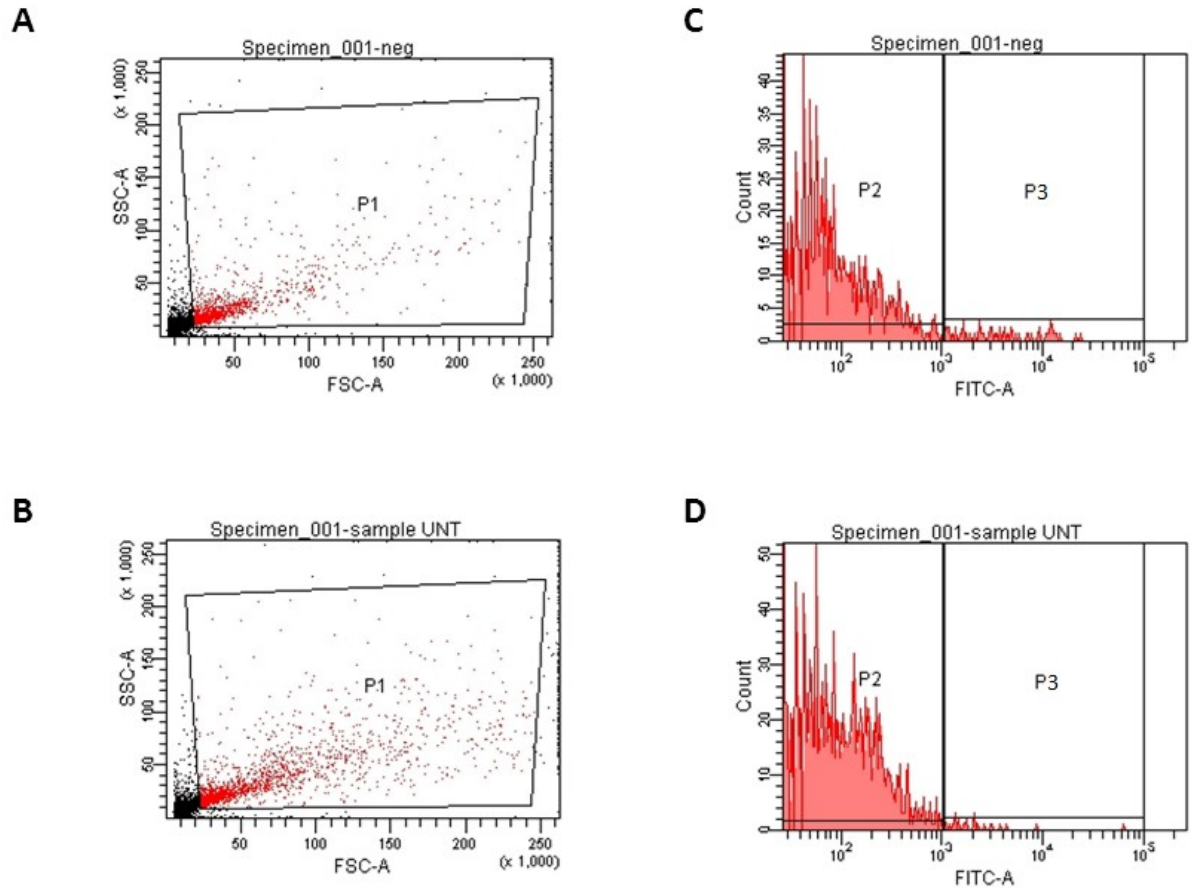


Figure 5.28 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the NHA cell lines pre hypoxia treatment. No fluorescence intensity was observed in the NHA cell line (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

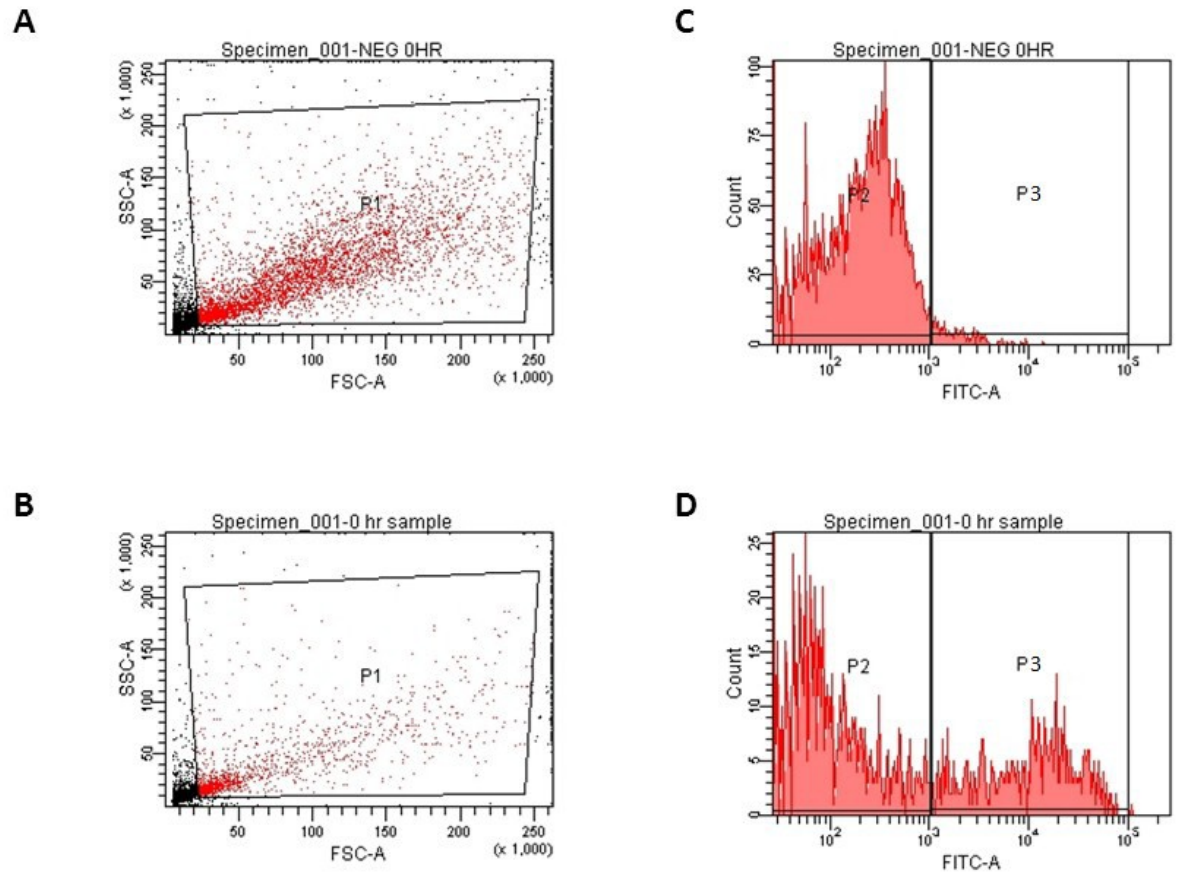


Figure 5.29 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the NHA cell lines post hypoxia treatment after 0 h recovery. Approximately 33% of NHA cells showed positive for HSPA. (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

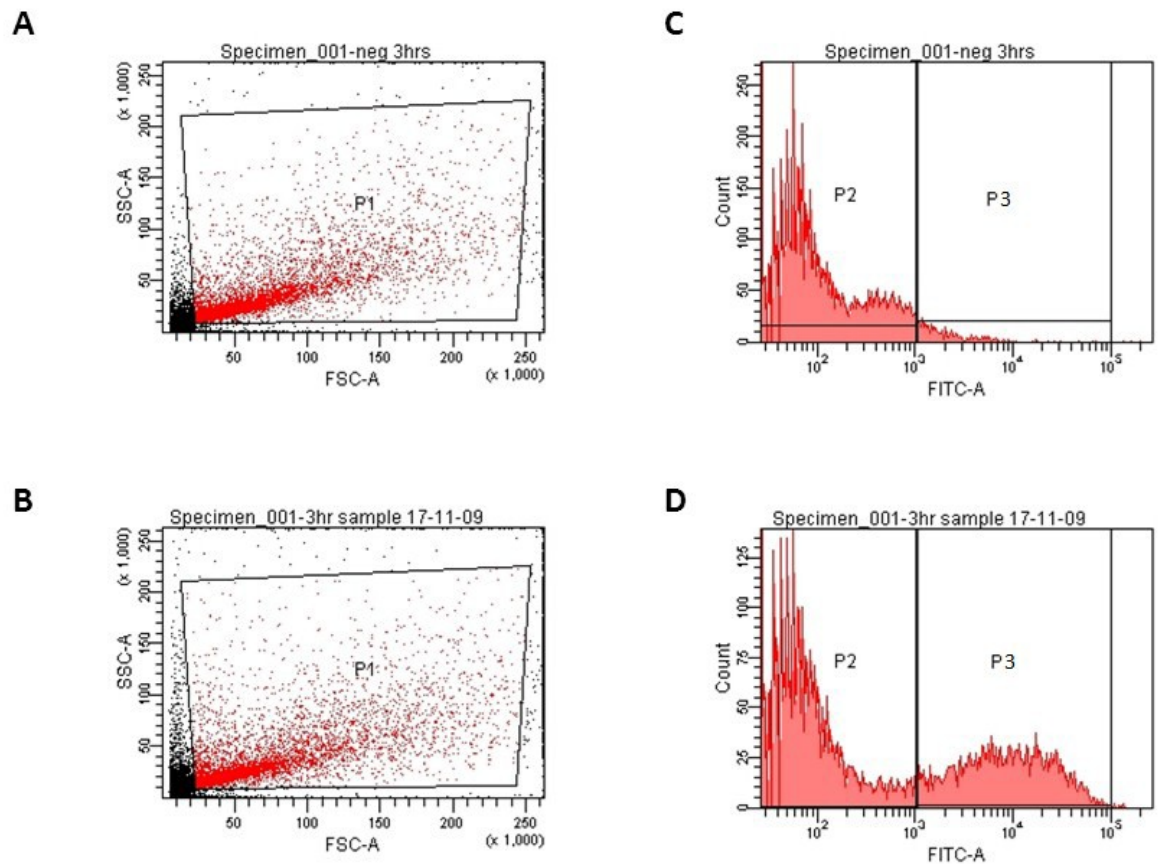


Figure 5.30 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the NHA cell lines post hypoxia treatment after 3 h recovery. Approximately 31% of NHA cells showed positive for HSPA. (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P5) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

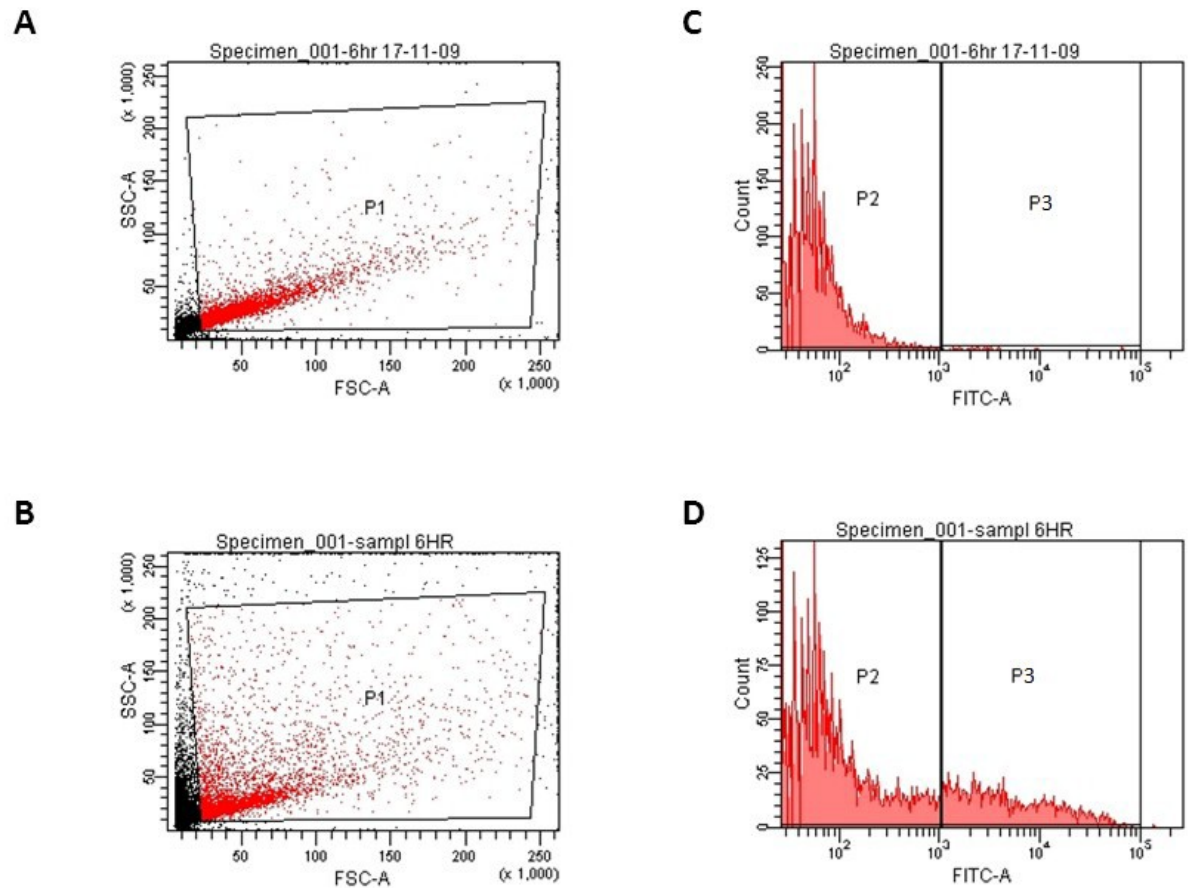


Figure 5.31 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the NHA cell lines post hypoxia treatment after 6 h recovery. Approximately 25% of NHA cells showed positive for HSPA. (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

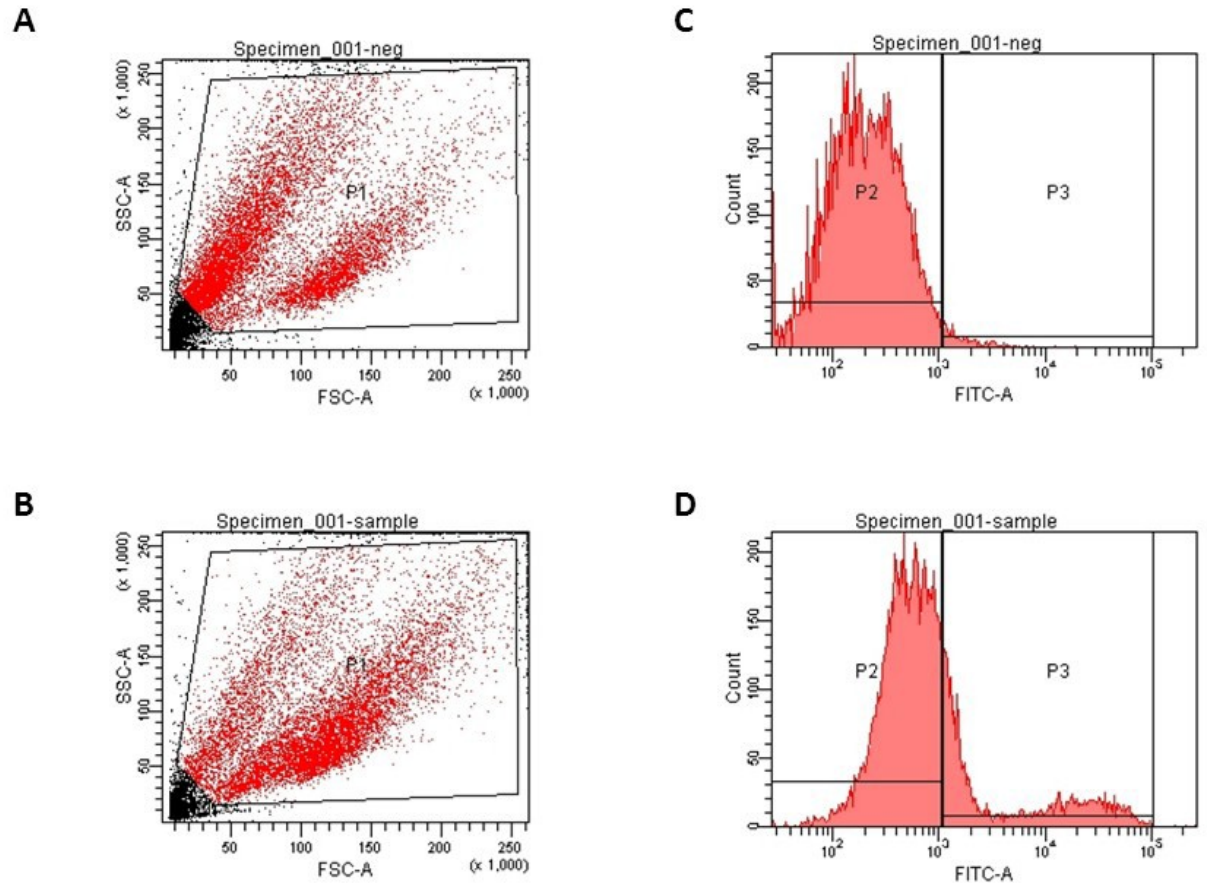


Figure 5.32 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the NHA cell lines post hypoxia treatment after 24 h recovery. Approximately 11% of NHA cells showed positive for HSPA. (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

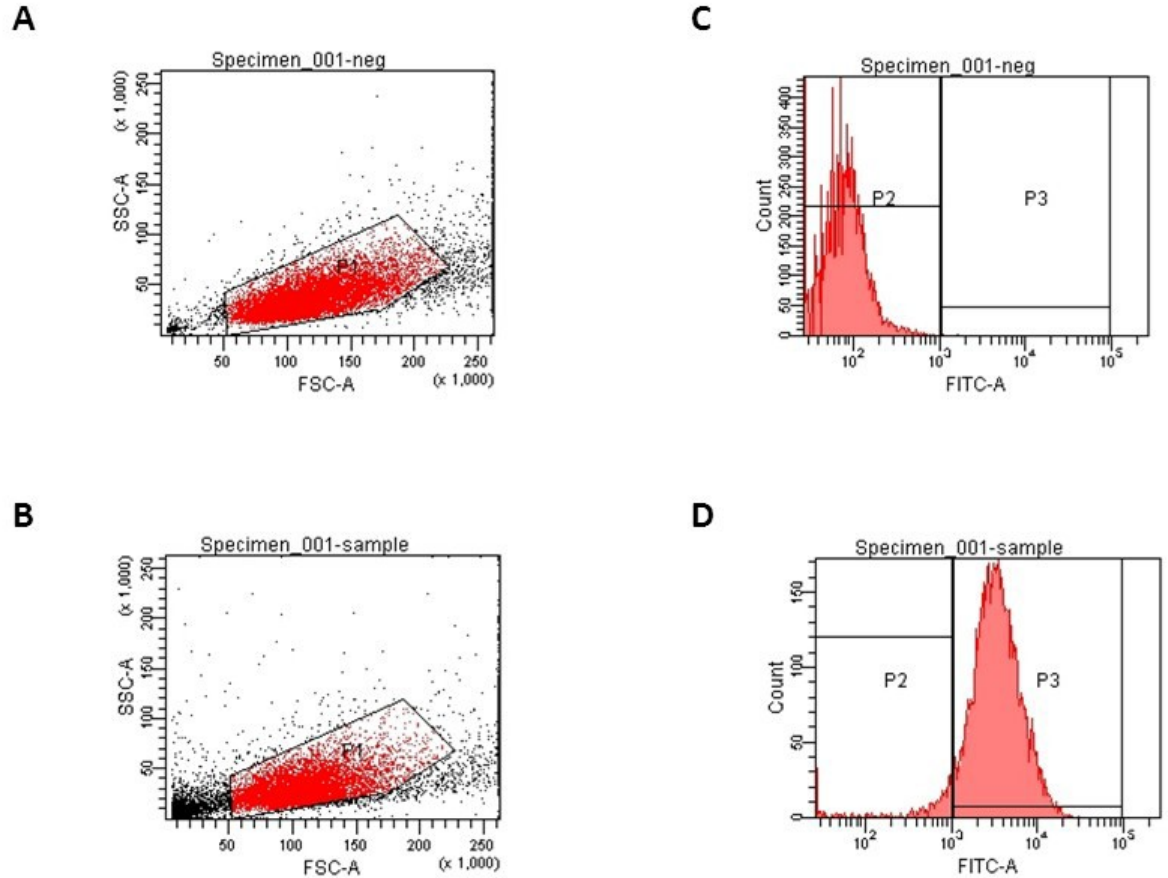


Figure 5.33 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the 1321N1 cell lines pre hypoxia treatment. Approximately 97% of 1321N1 glioma cells showed positive for HSPA. (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

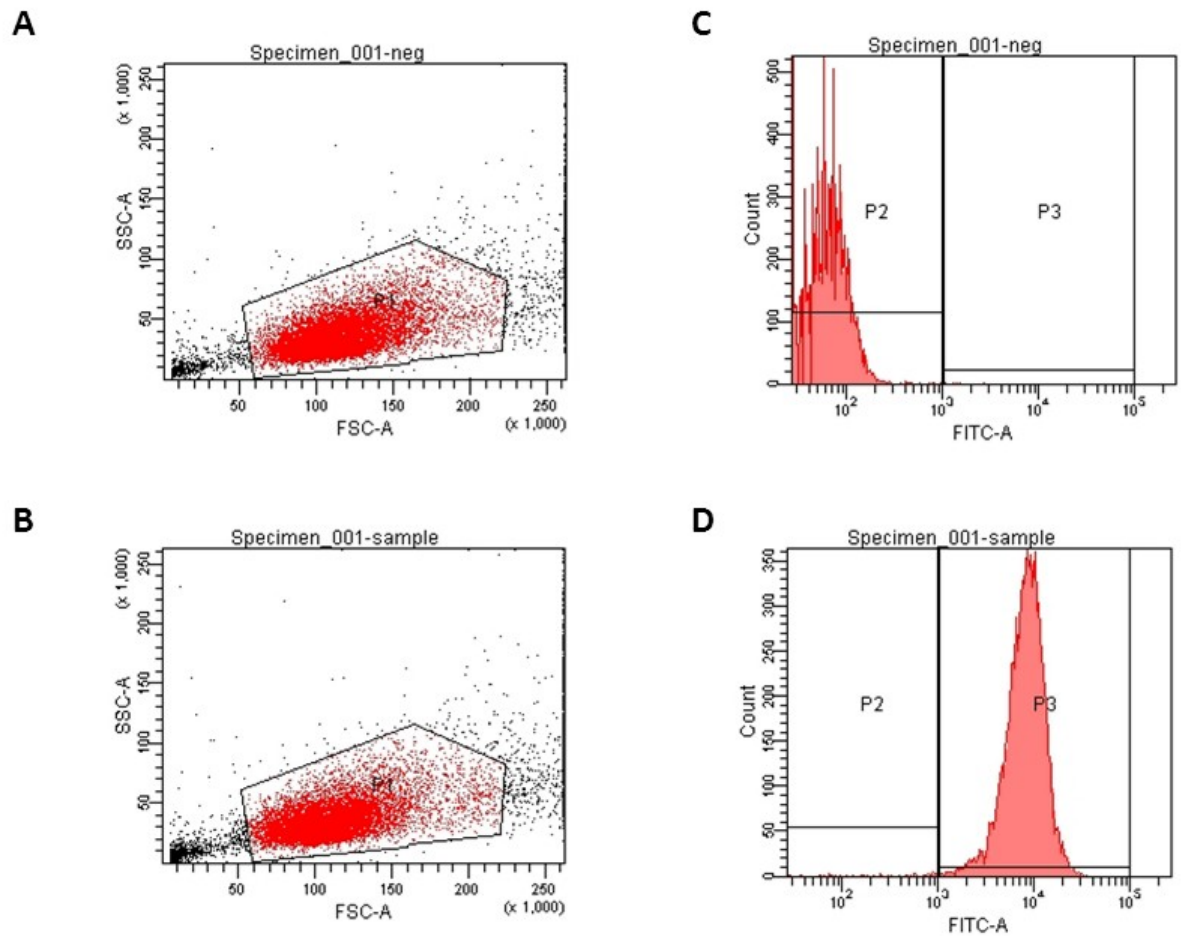


Figure 5.34 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the 1321N1 cell lines post hypoxia treatment after 0 h recovery. Approximately 99% of 1321N1 glioma cells showed positive for HSPA. (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

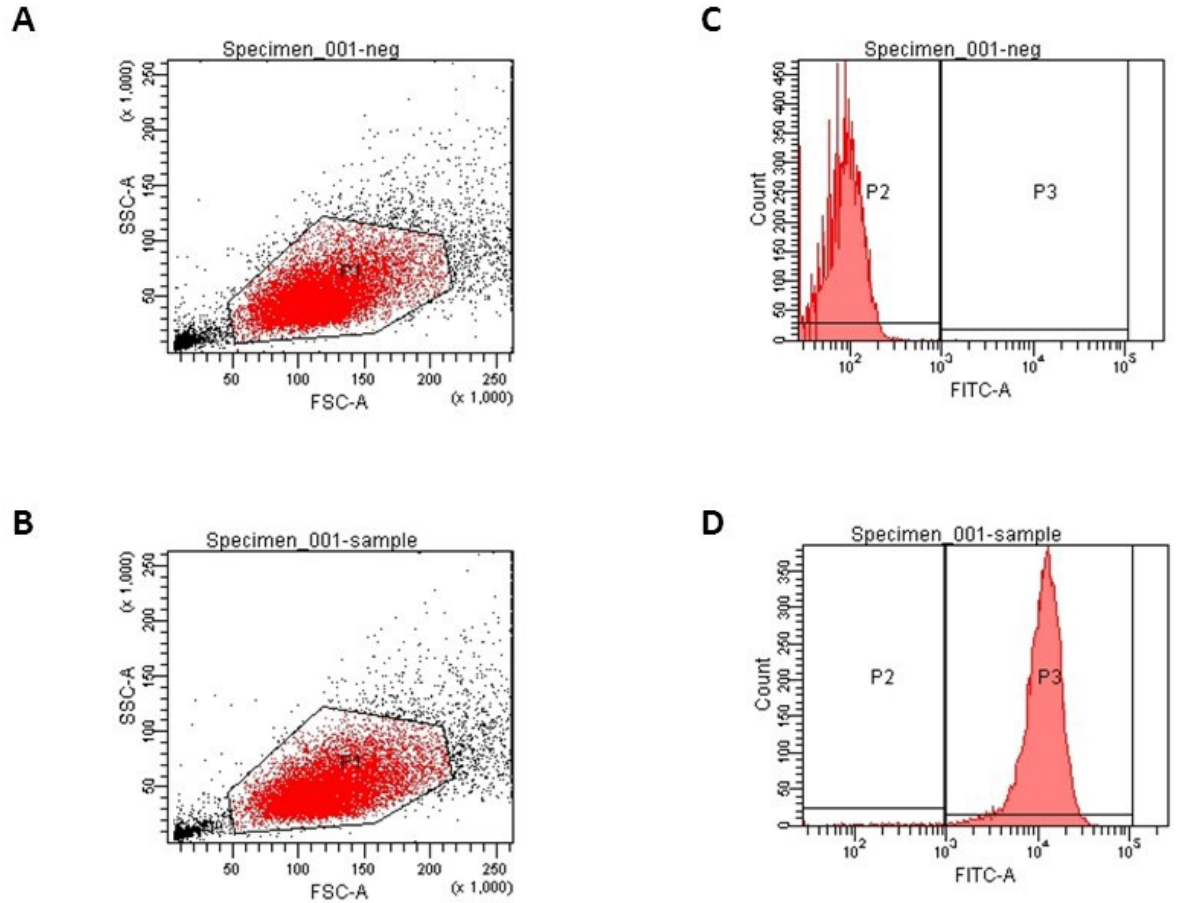


Figure 5.35 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the 1321N1 cell lines post hypoxia treatment after 3 h recovery. Approximately 99% of 1321N1 glioma cells showed positive for HSPA. (A, negative, primary antibody omitted and B, sample) 2D scatter plot showing cell population of interest (P1), (C, negative, D, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

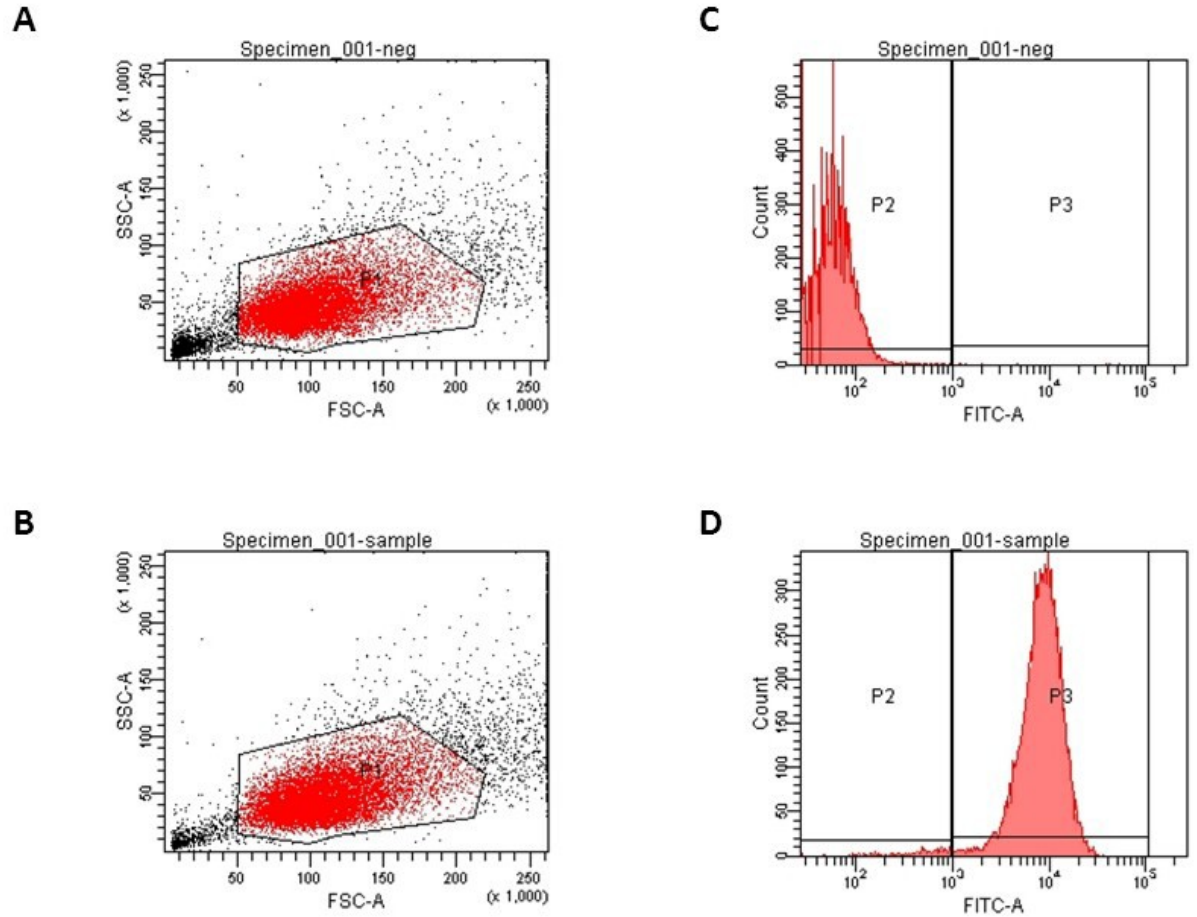


Figure 5.36 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the 1321N1 cell lines post hypoxia treatment after 6 h recovery. Approximately 99% of 1321N1 glioma cells showed positive for HSPA. (A, negative, primary antibody omitted and B, sample) 2D scatter plot showing cell population of interest (P1), (C, negative, D, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

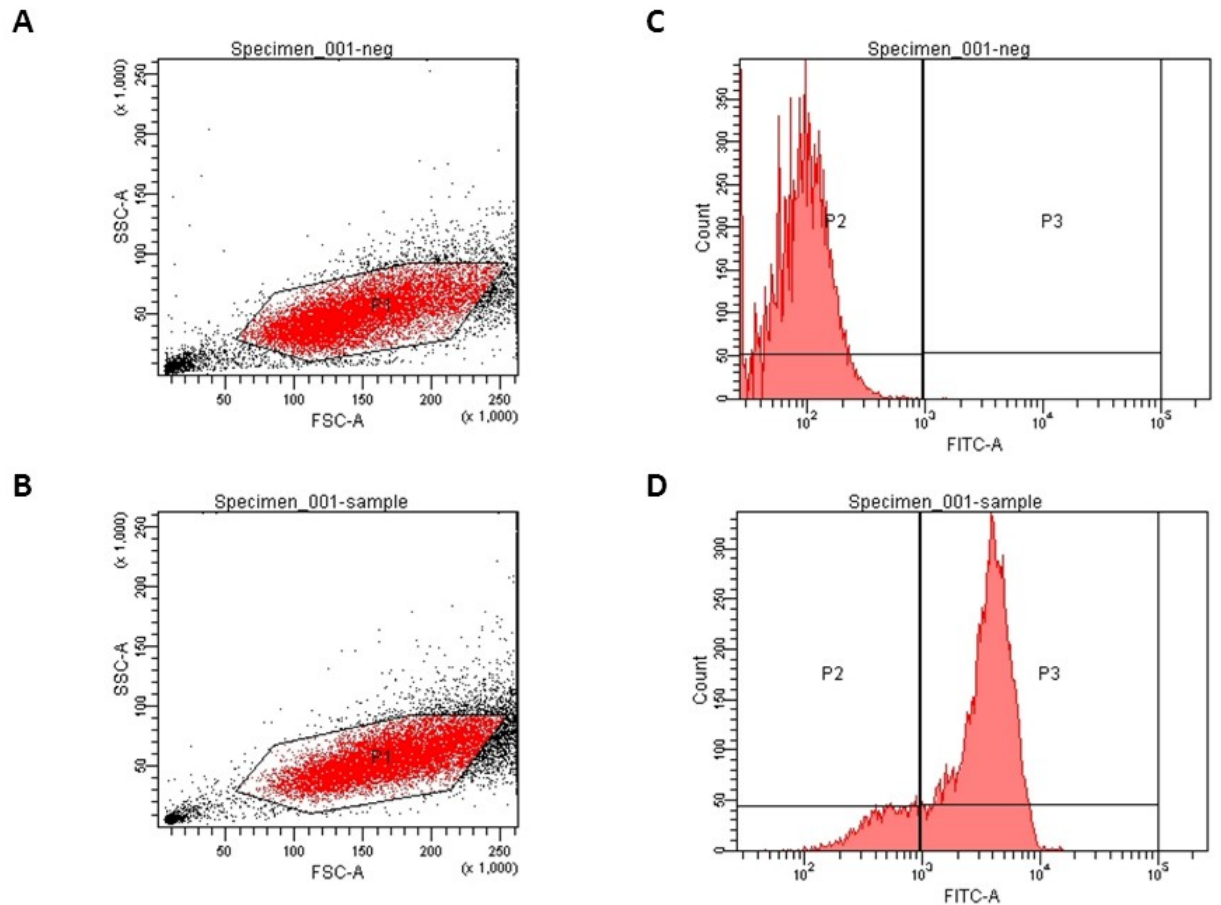


Figure 5.37 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the 1321N1 cell lines post hypoxia treatment after 24 h recovery. Approximately 94% of 1321N1 glioma cells showed positive for HSPA. (A, negative, primary antibody omitted and B, sample) 2D scatter plot showing cell population of interest (P1), (C, negative, D, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

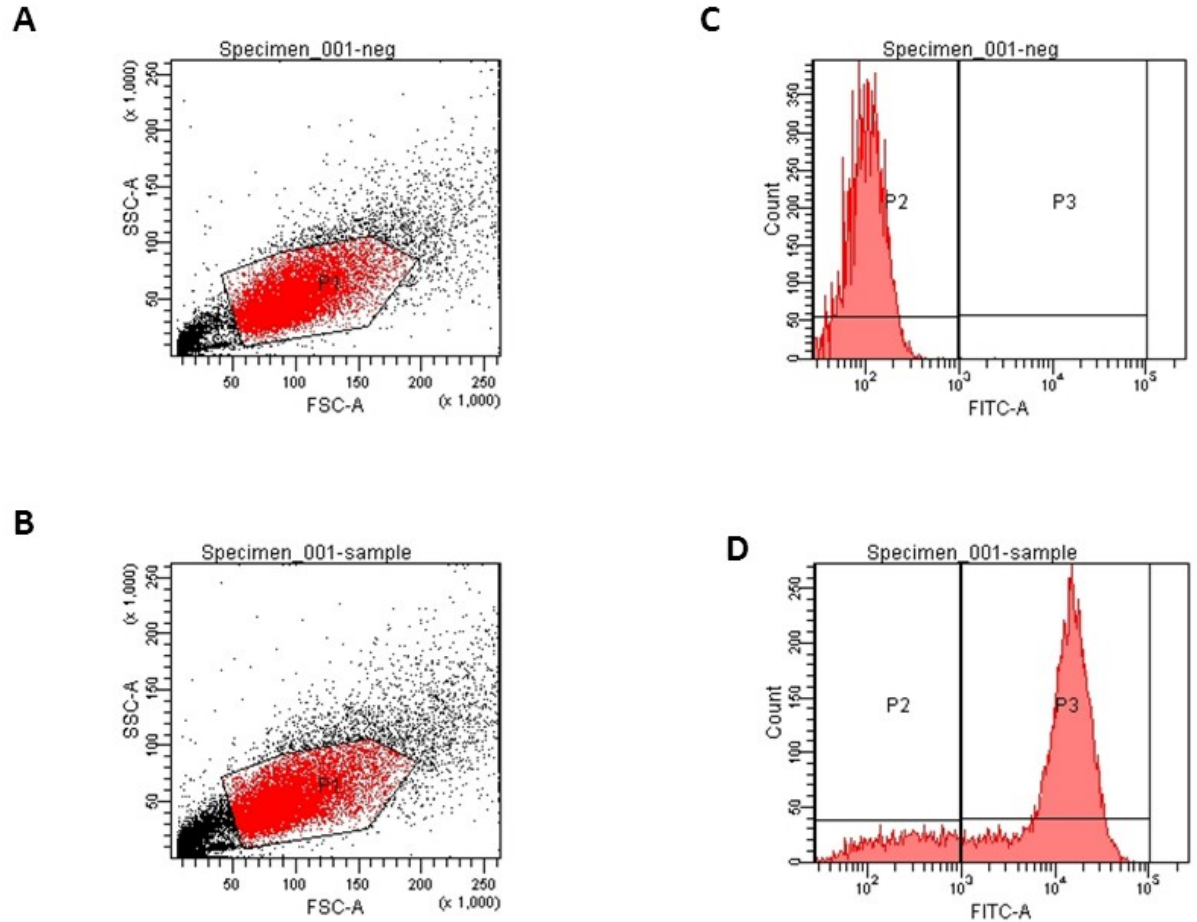


Figure 5.38 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the GOS-3 cell lines pre hypoxia treatment. Approximately 89% of GOS-3 glioma cells showed positive for HSPA. (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

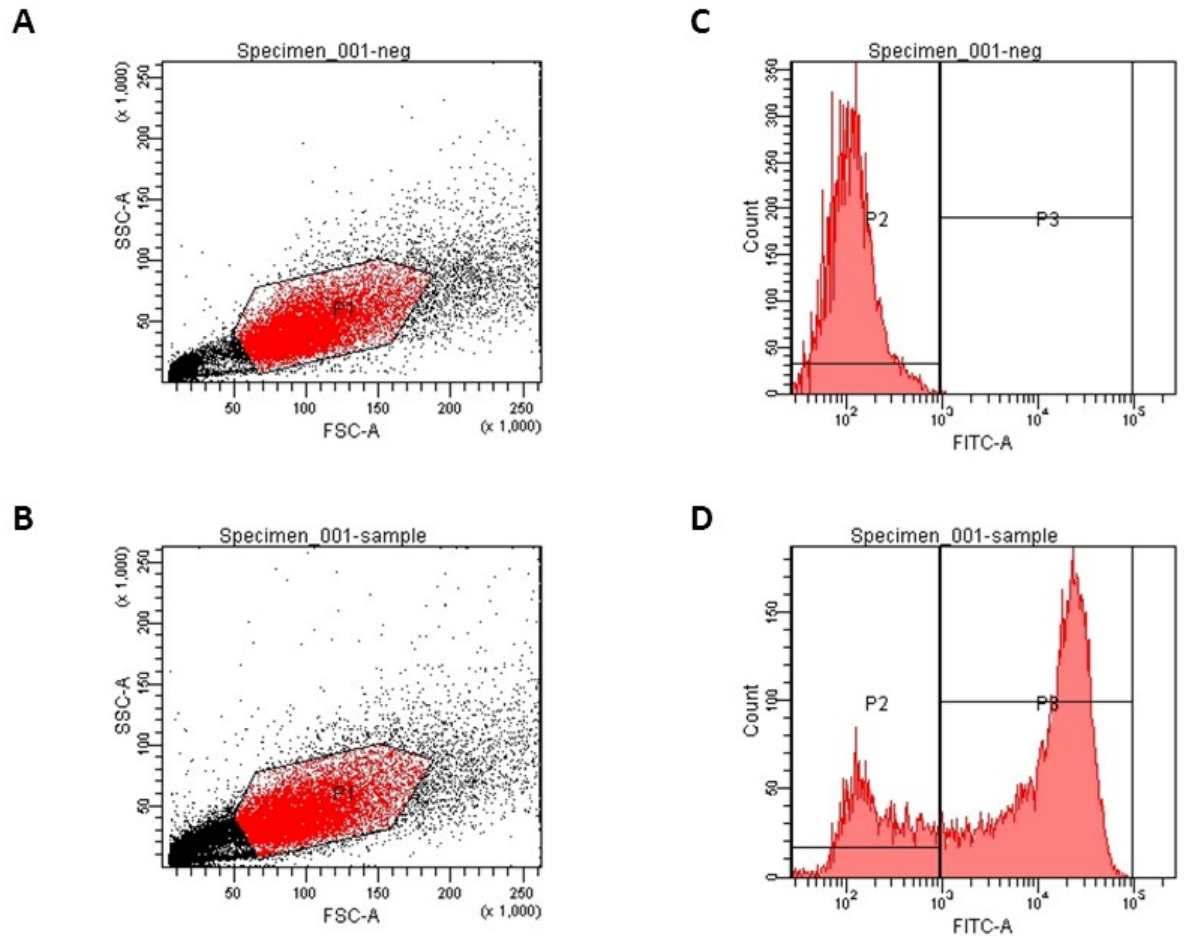


Figure 5.39 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the GOS-3 cell lines post hypoxia treatment after 0 h recovery. Approximately 73% of GOS-3 glioma cells showed positive for HSPA. (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

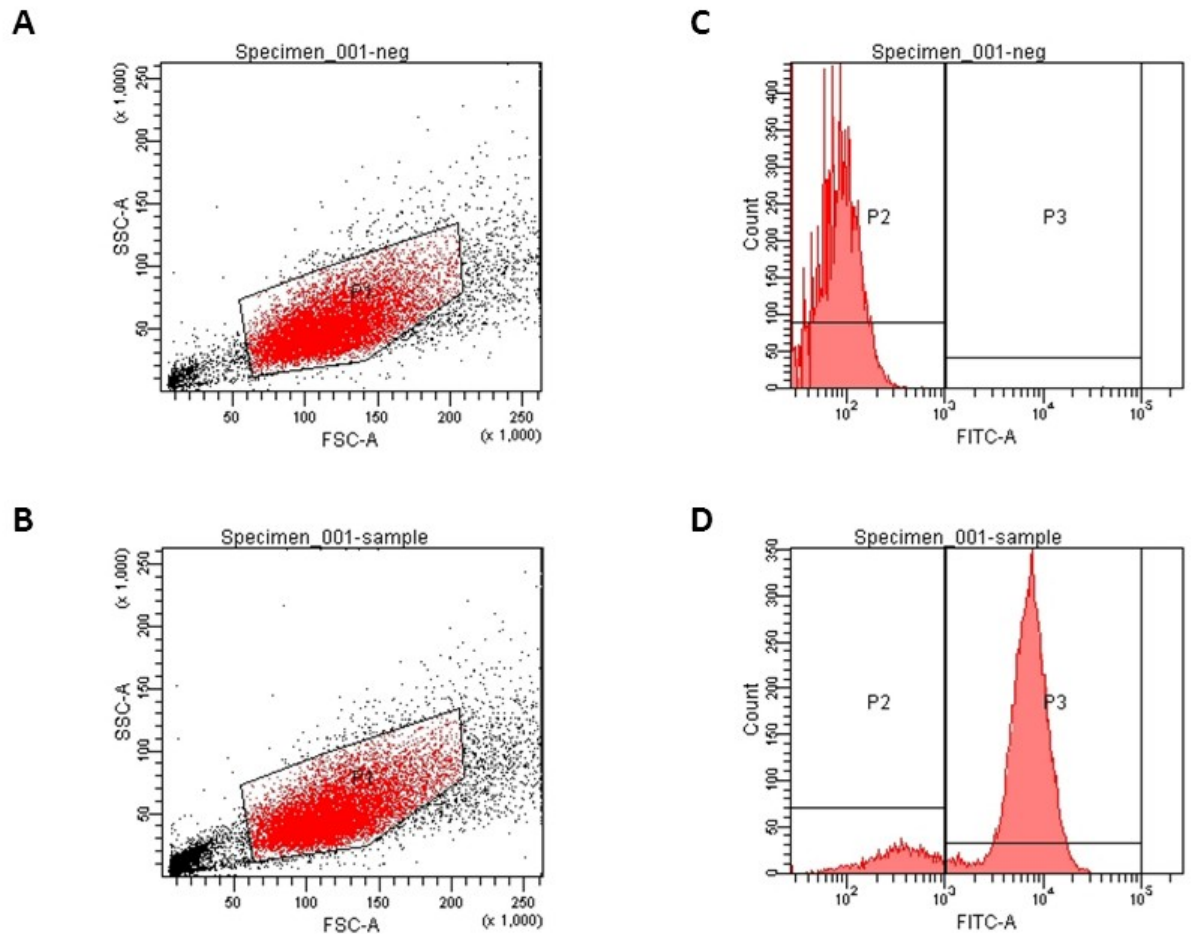


Figure 5.40 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the GOS-3 cell lines post hypoxia treatment after 3 h recovery. Approximately 92% of GOS-3 glioma cells showed positive for HSPA. (A, negative, primary antibody omitted and B, sample) 2D scatter plot showing cell population of interest (P1), (C, negative, D, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

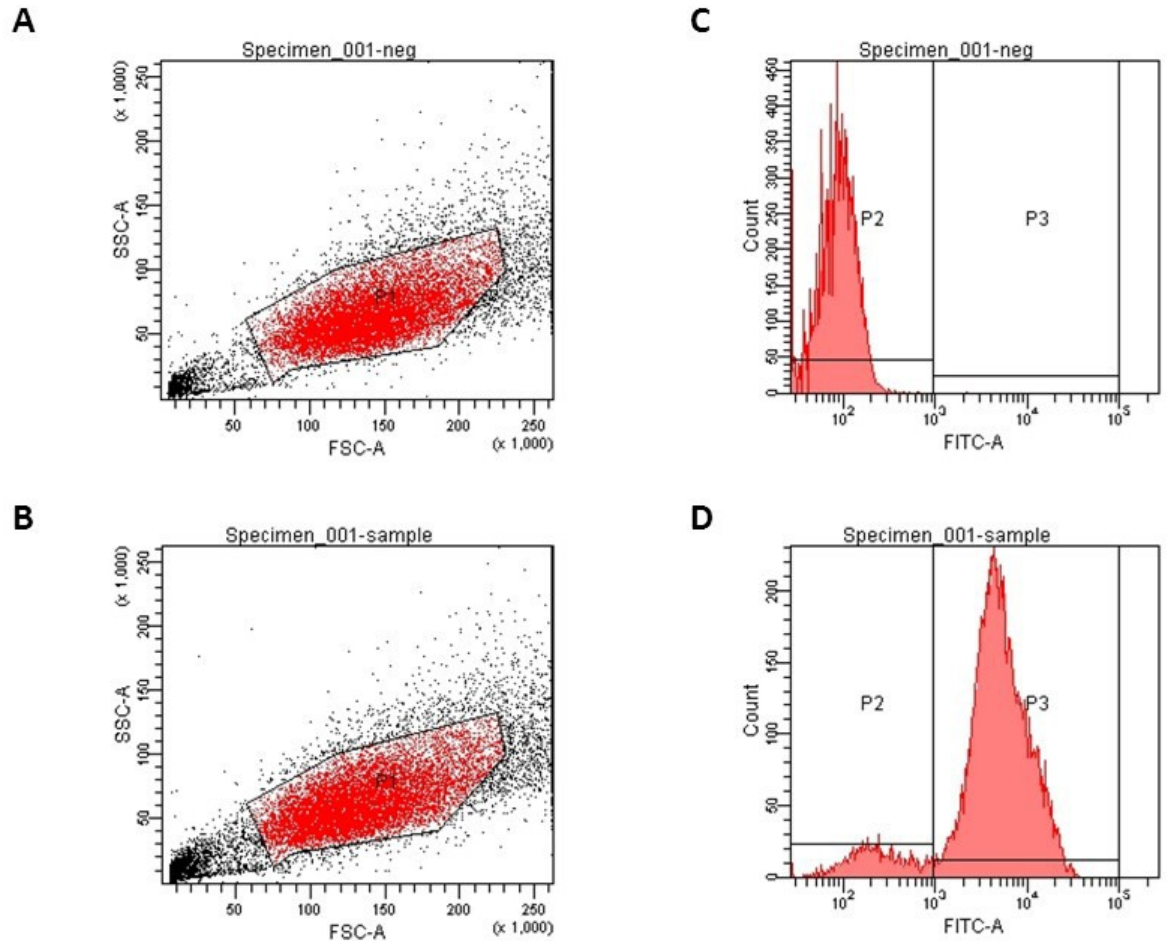


Figure 5.41 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the GOS-3 cell lines post hypoxia treatment after 6 h recovery. Approximately 92% of GOS-3 glioma cells showed positive for HSPA. (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

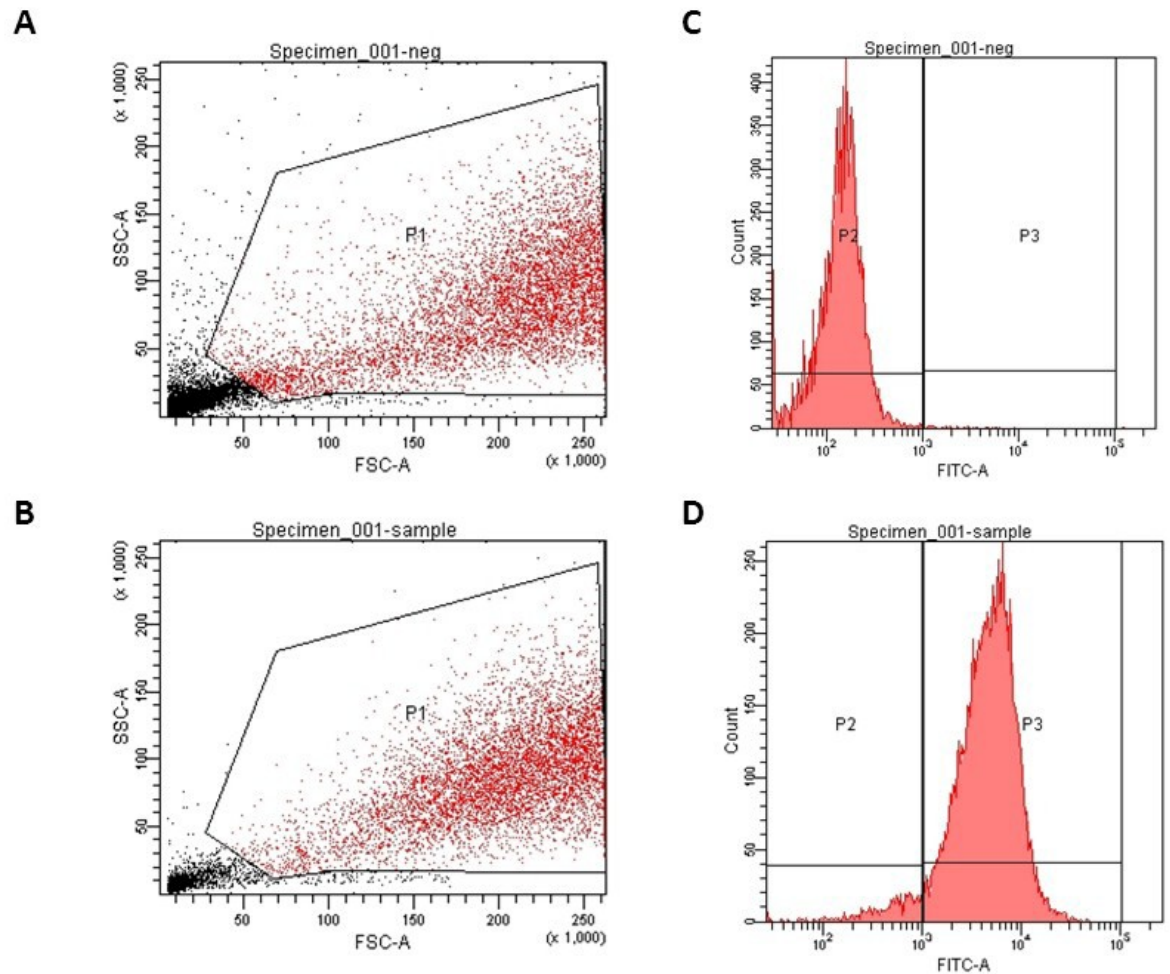


Figure 5.42 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the GOS-3 cell lines post hypoxia treatment after 24 h recovery. Approximately 97% of GOS-3 glioma cells showed positive for HSPA. (A, negative, primary antibody omitted and B, sample) 2D scatter plot showing cell population of interest (P1), (C, negative, D, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

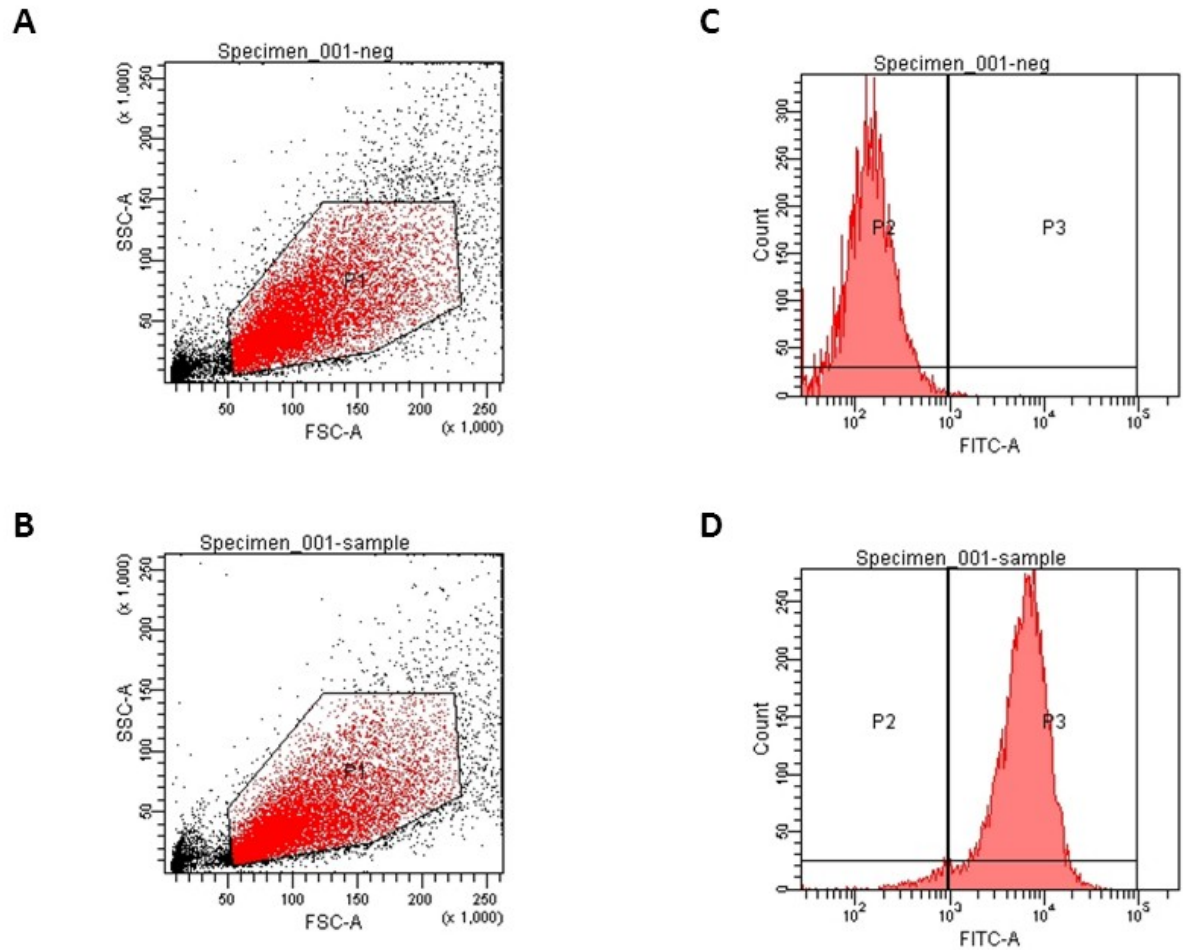


Figure 5.43 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the U87-MG cell lines pre hypoxia treatment. Approximately 97% of U87-MG glioma cells showed positive for HSPA. (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

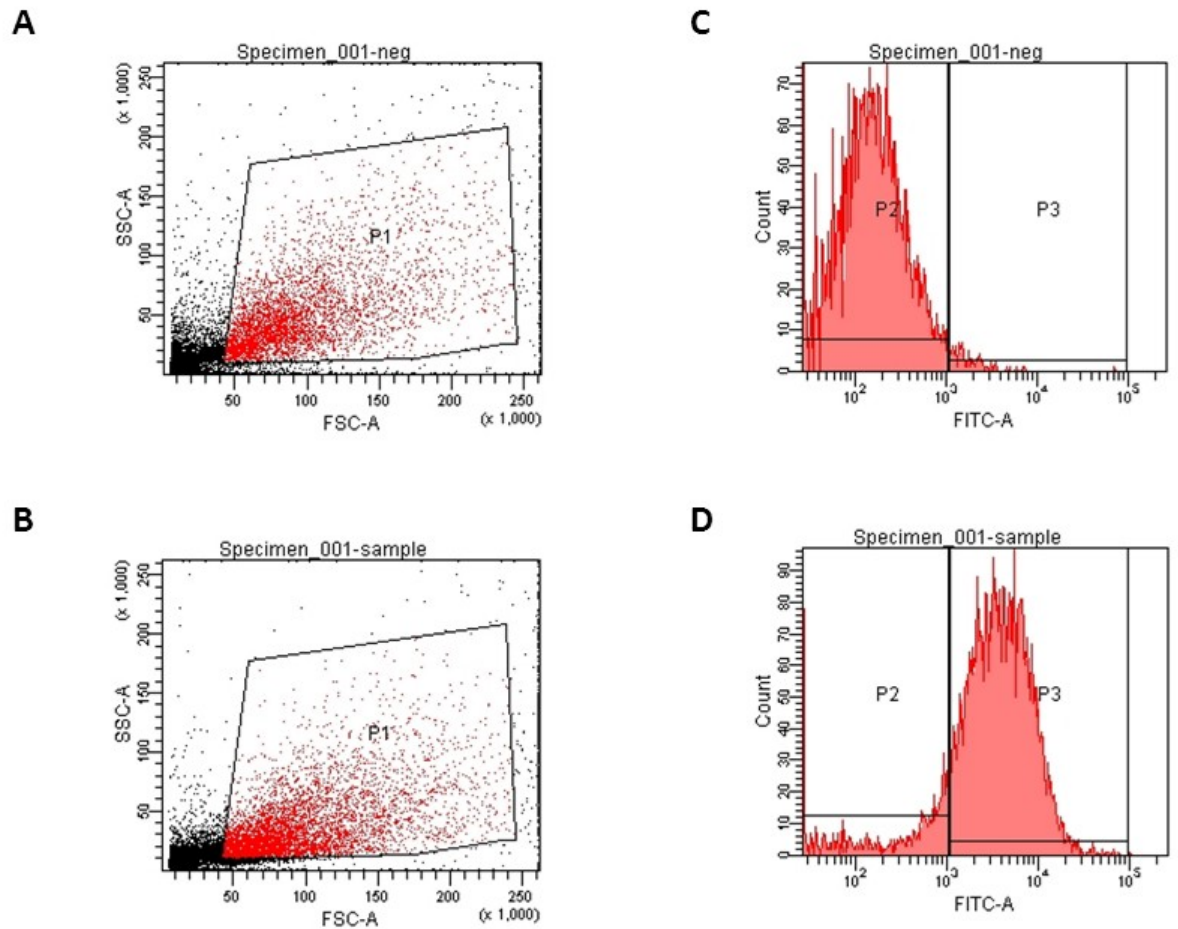


Figure 5.44 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the U87-MG cell lines post hypoxia treatment after 0 h recovery. Approximately 84% of U87-MG glioma cells showed positive for HSPA. (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

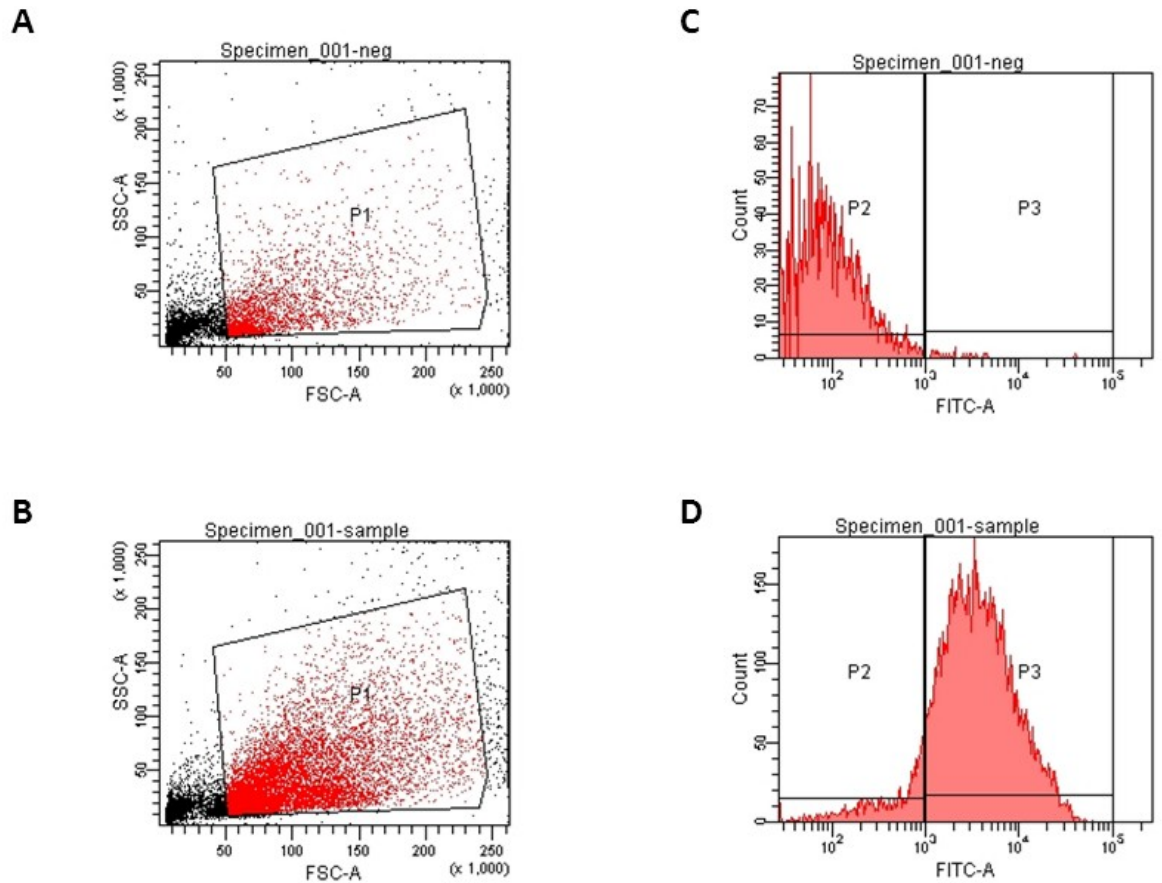


Figure 5.45 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the U87-MG cell lines post hypoxia treatment after 3 h recovery. Approximately 91% of U87-MG glioma cells showed positive for HSPA. (A, negative, primary antibody omitted and B, sample) 2D scatter plot showing cell population of interest (P1), (C, negative, D, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

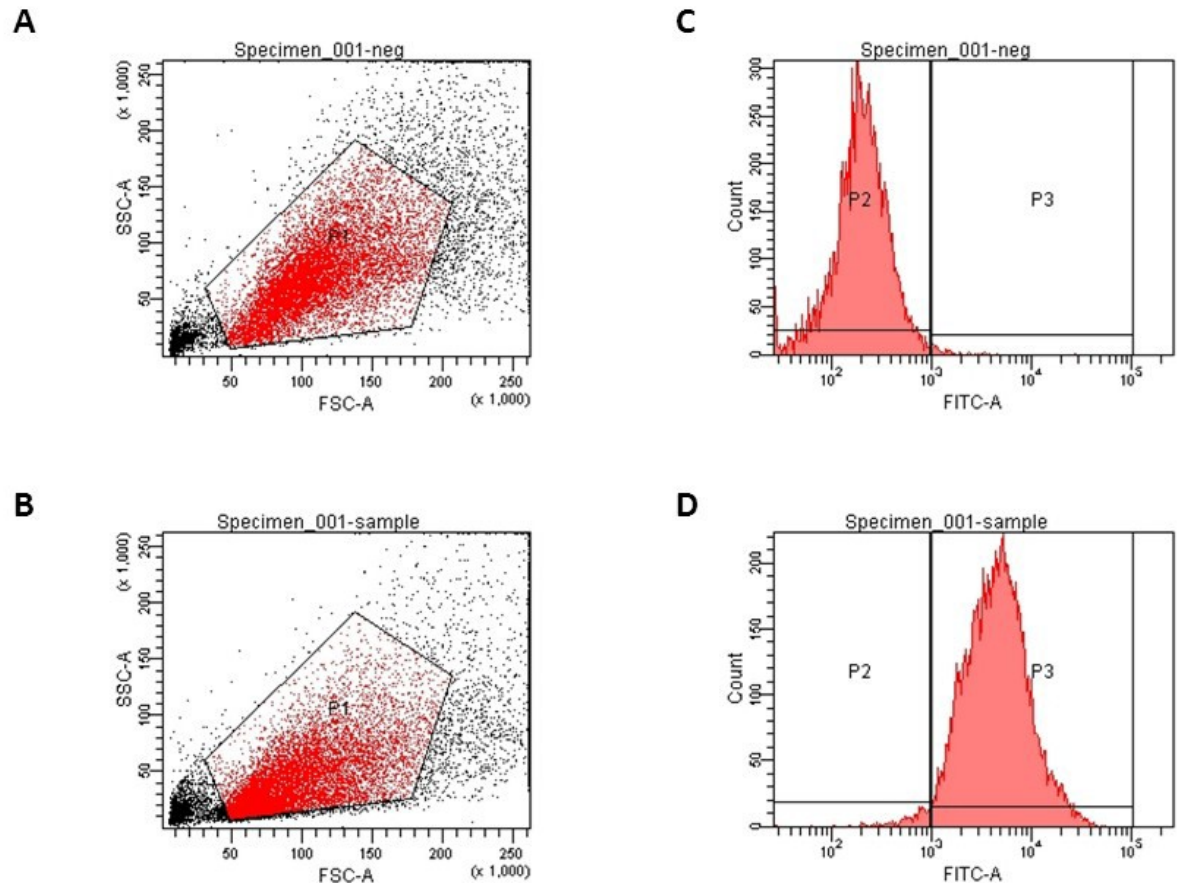


Figure 5.46 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the U87-MG cell lines post hypoxia treatment after 6 h recovery. Approximately 97% of U87-MG glioma cells showed positive for HSPA. (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

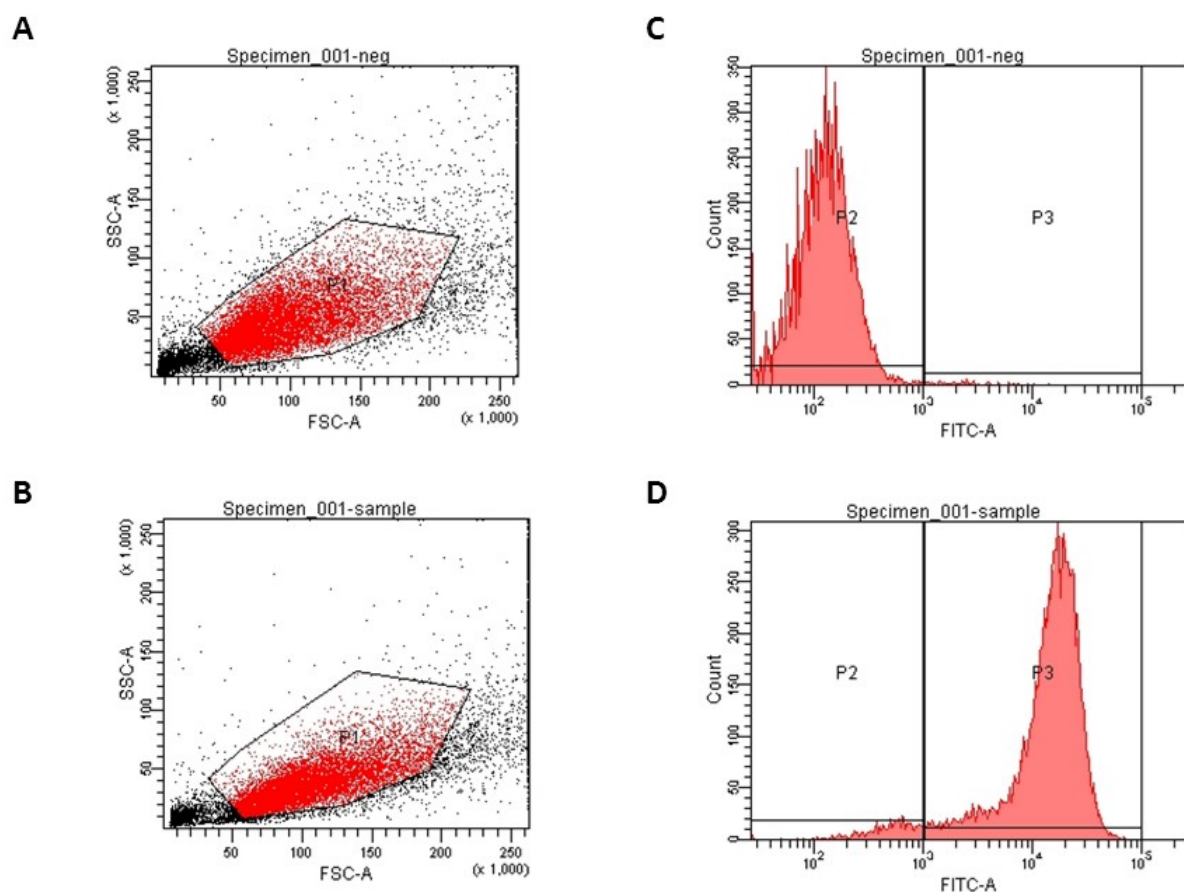


Figure 5.47 2D scatter plots and fluorescence intensity histograms of HSPA protein levels for the U87-MG cell lines post hypoxia treatment after 24 h recovery. Approximately 96% of U87-MG glioma cells showed positive for HSPA. (**A**, negative, primary antibody omitted and **B**, sample) 2D scatter plot showing cell population of interest (P1), (**C**, negative, **D**, sample) fluorescent intensity histogram showing cells positive (P3) and negative (P2) for HSPA. Data values are for three independent experiments considering 10,000 events per sample.

Results from this investigation show that *HSPA* is expressed at low levels in normal cells, but is highly expressed in cancer cell lines in pre- and post-hypoxia treatment. The average *HSPA* mRNA copy numbers in the three glioma cell lines for both pre- and post-hypoxia treatment were approximately 6 fold higher than the normal cell line.

As with *HSPA* mRNA, in both pre- and post-hypoxia treatment, high levels of HSPA protein were detected in the three glioma cell lines compared to low/barely detectable levels in the NHA cell line, indicating a direct correlation between *HSPA* mRNA and HSPA protein levels in glioma cell lines. High fluorescence intensity of HSPA observed in 1321N1, GOS-3 and U87-MG cells exposed to hypoxia showed a 3-fold increase compared to NHA cell line.

Again these results suggest that *HSPA* mRNA and HSPA protein levels in glioma may possibly be grade related, and therefore may be useful as a possible prognostic marker.

As expected, given that cancer is a form of cellular stress, HSPA protein was detected in both the cytoplasm and the nucleus of the three glioma cell lines under pre and post hypoxic conditions. Although HSPA protein was detected mainly in the cytoplasm in the normal (NHA) cell line pre-hypoxia, after hypoxia treatment migration of HSPA protein to the nucleus was observed.

Chapter 6

Effects of heat shock treatment on

***HSPA* gene expression in rat**

brain tissue

6.1 Gene Expression

HSPA expression is up-regulated in cells in response to heat shock and post-mortem conditions. The main research aims of this chapter was to measure and compare *HSPA* expression in normal and heat shocked post-mortem rat brain tissue and to characterise the possible “*de-novo*” expression of *HSPA* in response to severe heat shock.

Studies were undertaken using post-mortem brain tissue samples from aged-matched male Wistar rats (obtained from the Physiology Laboratory, University of Central Lancashire). All procedures conformed to the –UK Animals (Scientific Procedures) Act 1986 conforming to the –Principles of Laboratory Animal Care, 1985.

Rat brain tissue excised at 0, 3, 6, 12 and 24 h post sacrifice was subjected to mild heat shock at 0 h time course interval. For thermotolerance studies, brain tissue was subjected to mild heat shock at 0 h and again at 3, 6 and 24 h time course periods. Mild heat shock was conducted by immersion of the brain tissue, held in sterile 1.5 microfuge tubes, into a heated water bath at 42°C for a period of 10 min, after which brain tissue samples were stored at room temperature. Samples weighing approximately 50 mg were excised at timed intervals of 0, 3, 6, 12 and 24 h post sacrifice. Excised samples were immediately weighed and snap-frozen by immersion in liquid nitrogen, followed by grinding of the tissue to a fine powder using a sterile pestle and mortar, again under liquid nitrogen.

mRNA was isolated from post-mortem rat brain tissue, using mRNA Isolation Kit (Roche,UK) following the manufacturer’s protocol. The concentration and purity of mRNA was determined by ultraviolet spectrophotometry. Isolated mRNA (100 ng) was

transcribed to cDNA using 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche, UK) following the manufacturer's protocol, which was then used as a template for qRT-PCR. Quantitative real-time PCR was used to evaluate the expression of *HSPA* and GAPDH as a control using FastStart DNA Master^{PLUS} SYBR Green 1 (Roche, UK).

Primers used for *HSPA* were 5' GTGTGGAGAGCCAAGAGGAG 3' (sense) and 5' TTTCCAAACTGGATCGAAGG 3' (antisense). For GAPDH, primers were 5' AGACAGCCGCATCTTCTTGT 3' (sense) and 5' CTTGCCGTGGGTAGAGTCAT 3' (antisense). All primers were designed using Primer3 software and manufactured by TIB MOLBIOL.

After an initial denaturation at 95°C for 10 min, the samples were subjected to 35 cycles of RT-PCR 95°C for 10 s, annealing temperature 56°C (*HSPA*) and 57°C (GAPDH) for 15 s, and 72°C for 15 s (Patel *et al.*, 2008). At the end of each cycle, the fluorescence emitted was measured in a single step in channel F1 (gain1). After the 35th cycle, the specimens were heated to 95°C and rapidly cooled to 65°C for 15 s. All heating and cooling steps were performed with a slope of 20°C / s. The temperature was subsequently raised to 95°C with a slope of 0.1°C / s and fluorescence was measured continuously (channel F1, gain1) to obtain data for the melting curve analysis. All PCR reactions were performed in triplicate and a negative control included, which contained primers with no DNA. All PCR products were analysed using gel electrophoresis stained and visualised using a gel analyser (SynGene, UK).

Expression of *HSPA* was measured in rat post-mortem brain tissue at post-mortem intervals 0, 3, 6, 12 and 24 h and at time course intervals of 0, 3, 6, 12 and 24 h within each post-mortem interval. Post sacrifice, the brain was sectioned in two. Half was used for control (non-heat shock) and half was subjected to mild heat shock at 0 h time course for 10 min at 42°C.

The expression of *HSPA* was also measured in rat post-mortem brain tissue at post-mortem intervals 0, 3, 6 and 24 h and at time course intervals of 0, 3, 6 and 24 h within each post-mortem interval to access the effect of thermotolerance. Post sacrifice, the brain was sectioned in two, and half was used as a control (non-heat shock) and half was subjected to mild heat shock at 0 h time course for 10 min at 42°C, and then again at 3, 12 and 24 h time course intervals. The brain tissue both control and after heat shock at the various post-mortem intervals and time course was used for experiments concerning gene expression and immunofluorescence. *GAPDH* was used as an internal standard throughout all RT-PCR experiments (Barber *et al.*, 2005).

6.2 Constitutive Expression

6.2.1 Rat Brain tissue

Transcript copy numbers of *HSPA* and *GAPDH* were evaluated using RT-PCR in rat post-mortem brain tissue at post-mortem intervals 0, 3, 6, 12 and 24 h and at time course intervals of 0, 3, 6, 12 and 24 h within each post-mortem interval, for both control (non-heat shock) and mild heat shock treatment samples. The primers and optimal temperatures used for the amplification of both genes are documented in Table 2.10 in section 2.5. All PCR experiments were carried out in triplicate for consistency and repeatability. For each gene analysed, a quantification graph was produced to confirm gene amplification. The resulting amplicons for *HSPA* and *GAPDH* were then visualized using agarose gel electrophoresis, when they were represented by bands of 156 and 265 bp respectively.

At 0 h and 3 h post-mortem interval at 0, 3, 6, 12 and 24 h time course, heat shocked brain tissue contained *HSPA* transcripts at a higher level compared to that of the non-

heat shocked brain tissue (Figure 6.1 and 6.2). At 6 h post-mortem interval *HSPA* transcribed higher in heat shocked brain tissue at time course 0, 3, 12 and 24 h, but lower at the 6 h time course than non-heat shocked brain tissue (Figure 6.3). At 12 h post-mortem interval *HSPA* transcribed higher in heat shocked brain tissue at time course 0 and 12 h, but lower at the 3, 6 and 24 h time course than non-heat shocked brain tissue (Figure 6.4). At 24 h post-mortem interval *HSPA* transcribed higher in heat shocked brain tissue at time course intervals; 0, 3 and 24 h, but lower at the 6 h and 12 h time course intervals than non-heat shocked brain tissue (Figure 6.5).

GAPDH expression level remained relatively consistent throughout each set of experiments confirming it as an ideal reference gene (Figures 6.6 – 6.10).

The mRNA copy number per 100 ng of extracted mRNA for both genes was calculated for each time course 0, 3, 6, 12 and 24 h within each post-mortem interval 0, 3, 6, 12 and 24 h for both non-heat shock and heat shock to monitor the gene expression level. mRNA copy numbers confirmed that at: 0 h post-mortem interval, *HSPA* was expressed at a higher level in heat shocked brain tissue at different time course intervals, with approximately 21 copies after 0 h recovery, with approximately 52 copies after 3 h recovery, with approximately 83 copies after 6 h recovery, with approximately 55 copies after 12 h recovery and approximately 31 copies after 24 h recovery compared to non-heat shocked brain tissue at different time course intervals, with approximately 19 copies after 0 h recovery, with approximately 38 copies after 3 h recovery, with approximately 55 copies after 6 h recovery, with approximately 36 copies after 12 h recovery and approximately 17 copies after 24 h recovery (Figure 6.1 and Table 6.1).

At 3 h post-mortem interval, *HSPA* was expressed at a higher level in heat shocked brain tissue at different time course intervals, with approximately 58 copies after 0 h

recovery, with approximately 53 copies after 3 h recovery, with approximately 15 copies after 6 h recovery, with approximately 45 copies after 12 h recovery and approximately 42 copies after 24 h recovery compared with non-heat shocked brain tissue at different time course intervals, with approximately 17 copies after 0 h recovery, with approximately 37 copies after 3 h recovery, with approximately 9 copies after 6 h recovery, with approximately 35 copies after 12 h recovery and approximately 36 copies after 24 h recovery (Figure 6.2 and Table 6.1).

At 6 h post-mortem interval, *HSPA* was expressed at a higher level in heat shocked brain tissue at different time course intervals, with approximately 19 copies after 0 h recovery, with approximately 34 copies after 3 h recovery, with approximately 21 copies after 12 h recovery and approximately 37 copies after 24 h recovery compared to non-heat shocked brain tissue at different time course intervals, with approximately 10 copies after 0 h recovery, with approximately 12 copies after 3 h recovery, with approximately 3 copies after 12 h recovery and approximately 17 copies after 24 h recovery. Expression was lower in heat shocked brain tissue after 6 h recovery, approximately 9 copies compared to non-heat shocked brain tissue after 6 h recovery, approximately 20 copies (Figure 6.3 and Table 6.1).

At 12 h post-mortem interval, *HSPA* was expressed at a higher level in heat shocked brain tissue at different time course intervals, with approximately 25 copies after 0 h recovery and approximately 67 copies after 12 h recovery compared to non-heat shocked brain tissue at different time course intervals, with approximately 18 copies after 0 h recovery and approximately 17 copies after 12 h recovery. Expression was lower in heat shocked brain tissue at different time course intervals, with approximately 38 copies after 3 h recovery, with approximately 23 copies after 6 h recovery and

approximately 30 copies after 24 h recovery compare to non-heat shocked brain tissue at different time course intervals, with approximately 57 copies after 3 h recovery, with approximately 65 copies after 6 h recovery and approximately 40 copies after 24 h recovery (Figure 6.4 and Table 6.1).

At 24 h post-mortem interval, *HSPA* was expressed at a higher level in heat shocked brain tissue at different time course intervals, with approximately 25 copies after 0 h recovery, with approximately 27 copies after 3 h recovery, with approximately 20 copies after 6 h recovery and approximately 23 copies after 24 h recovery compare to non-heat shocked brain tissue at different time course intervals, with approximately 12 copies after 0 h recovery, with approximately 2 copies after 3 h recovery, with approximately 13 copies after 6 h recovery and approximately 9 copies after 24 h recovery. Expression was lower in heat shocked brain tissue after 12 h recovery, approximately 4 copies compared to non-heat shocked brain tissue after 12 h recovery, approximately 4 copies (Figure 6.5, Table 6.1).

For *GAPDH*, mRNA copy numbers per 100 ng of extracted mRNA were relatively consistent throughout each PMI, time course in both non-heat shock and heat shocked brain tissue, with approximately 268,500 copies for non-heat shock and approximately 267,300 copies for heat shock, again confirming comparability of *HSPA* results (Figures 6.6 – 6.10 and Table 6.2).

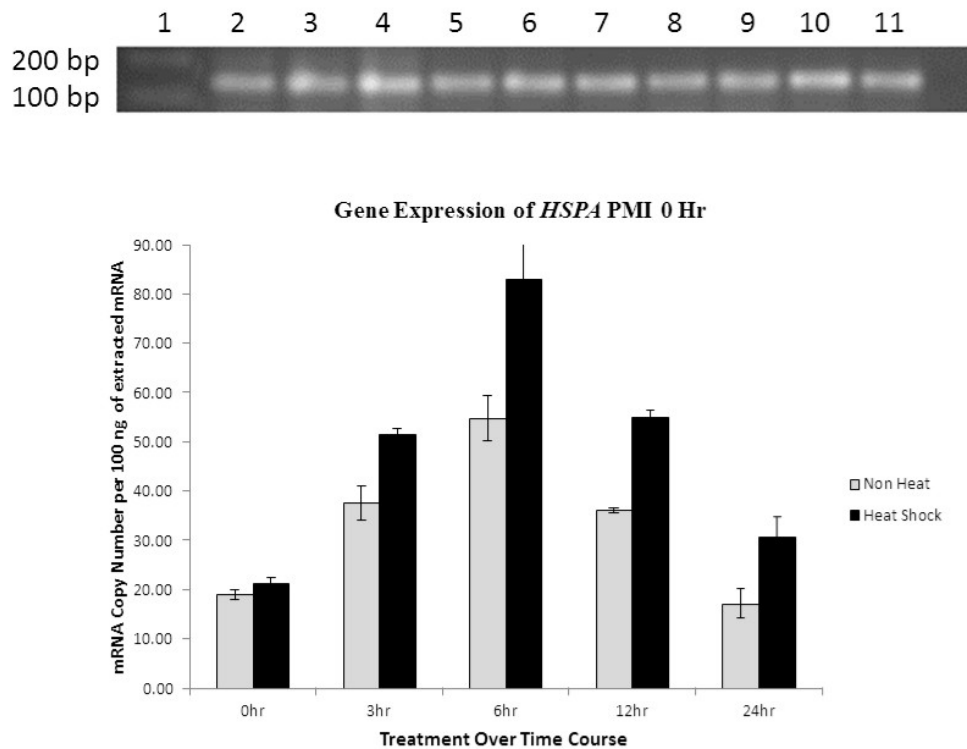


Figure 6.1 Transcription levels of *HSPA* mRNA in non-heat shock and heat shocked rat brain tissue at 0 h PMI. (A) agarose gel electrophoresis: Lane 1 represents the 100 bp molecular ladder; lanes 2, 4, 6, 8 and 10 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. Lanes 3, 5, 7, 9 and 11 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. (B) Histogram showing *HSPA* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6, 12 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars.

Control samples show post-mortem effect, as *HSPA* transcripts increase over time.

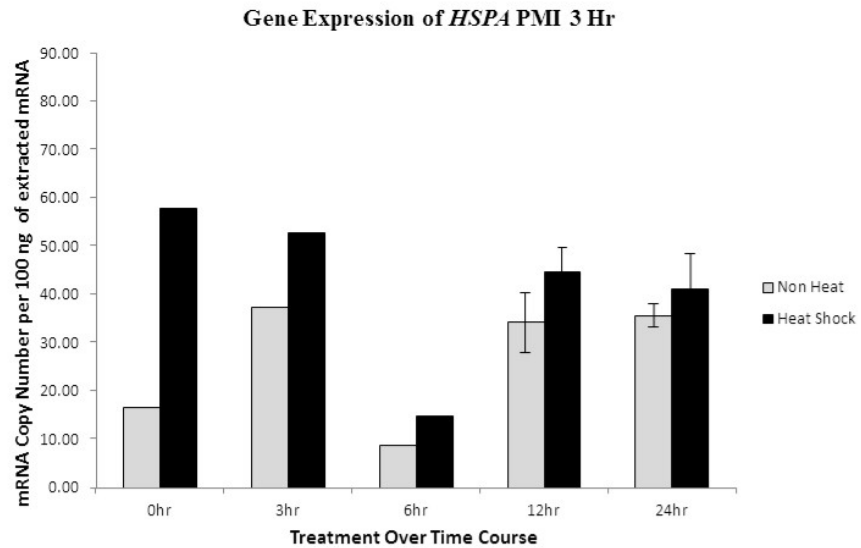
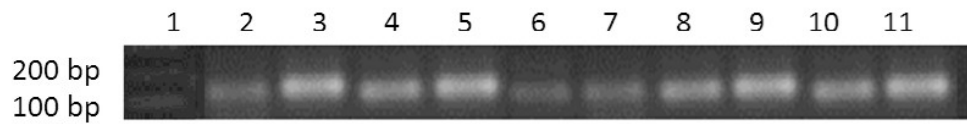


Figure 6.2 Transcription levels of *HSPA* mRNA in non-heat shock and heat shocked rat brain tissue at 3 h PMI. (A) agarose gel electrophoresis: Lane 1 represents the 100 bp molecular ladder; lanes 2, 4, 6, 8 and 10 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. Lanes 3, 5, 7, 9 and 11 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. (B) Histogram showing *HSPA* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6, 12 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars. For both non-heat shock and heat shocked samples at time course intervals 0, 3 and 6 h no error bars are present as a 0 value standard deviation was calculated.

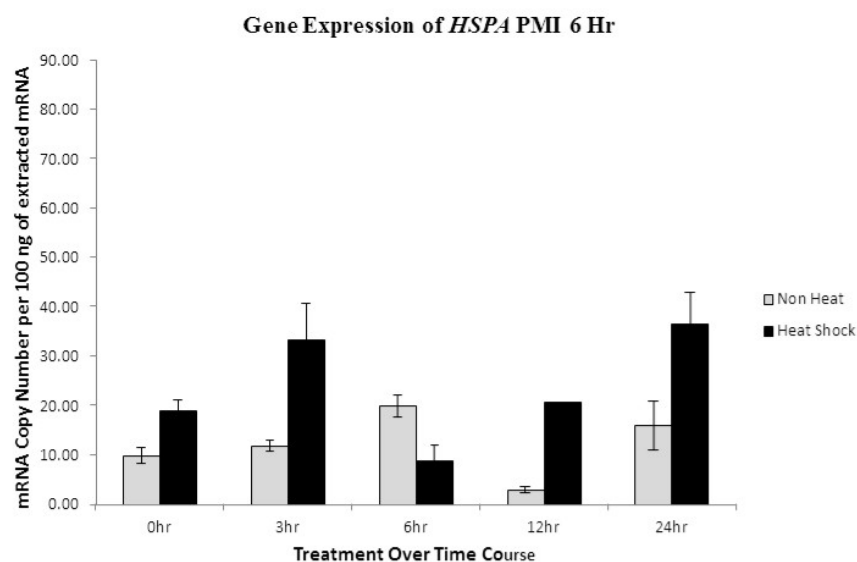
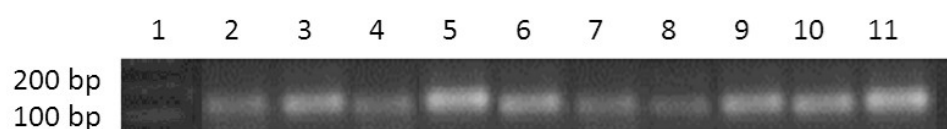


Figure 6.3 Transcription levels of *HSPA* mRNA in non-heat shock and heat shocked rat brain tissue at 6 h PMI. (A) Agarose gel electrophoresis: Lane 1 represents the 100 bp molecular ladder; lanes 2, 4, 6, 8 and 10 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. Lanes 3, 5, 7, 9 and 11 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. (B) Histogram showing *HSPA* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6, 12 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars.

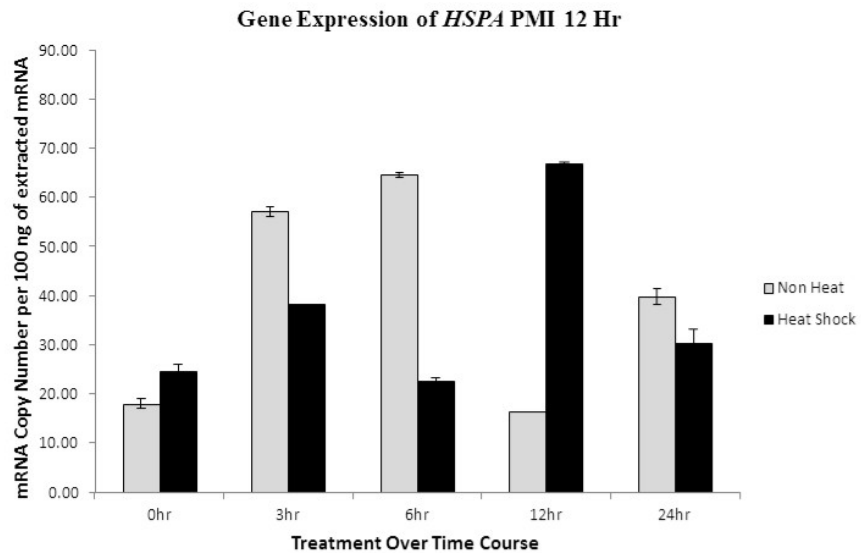
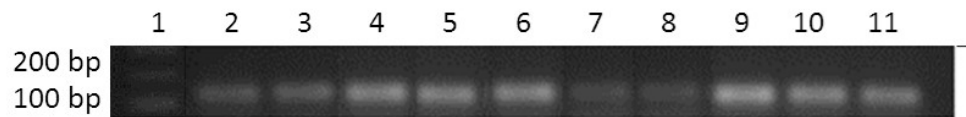


Figure 6.4 Transcription levels of *HSPA* mRNA in non-heat shock and heat shocked rat brain tissue at 12 h PMI. (A) Agarose gel electrophoresis: Lane 1 represents the 100 bp molecular ladder; lanes 2, 4, 6, 8 and 10 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. Lanes 3, 5, 7, 9 and 11 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. (B) Histogram showing *HSPA* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6, 12 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars. At 3 h heat shock and 12 h non-heat shock no error bars are present as a 0 value standard deviation was calculated.

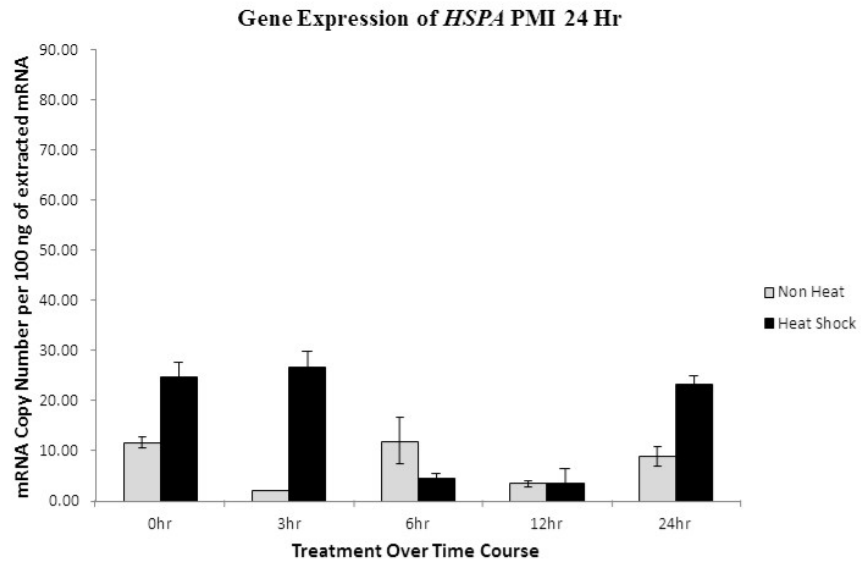


Figure 6.5 Transcription levels of *HSPA* mRNA in non-heat shock and heat shocked rat brain tissue at 24 h PMI. (A) Agarose gel electrophoresis: Lane 1 represents the 100 bp molecular ladder; lanes 2, 4, 6, 8 and 10 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. Lanes 3, 5, 7, 9 and 11 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. (B) Histogram showing *HSPA* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6, 12 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars. At 3 h non-heat shock and 6 h heat shock no error bars are present as a 0 value standard deviation was calculated.

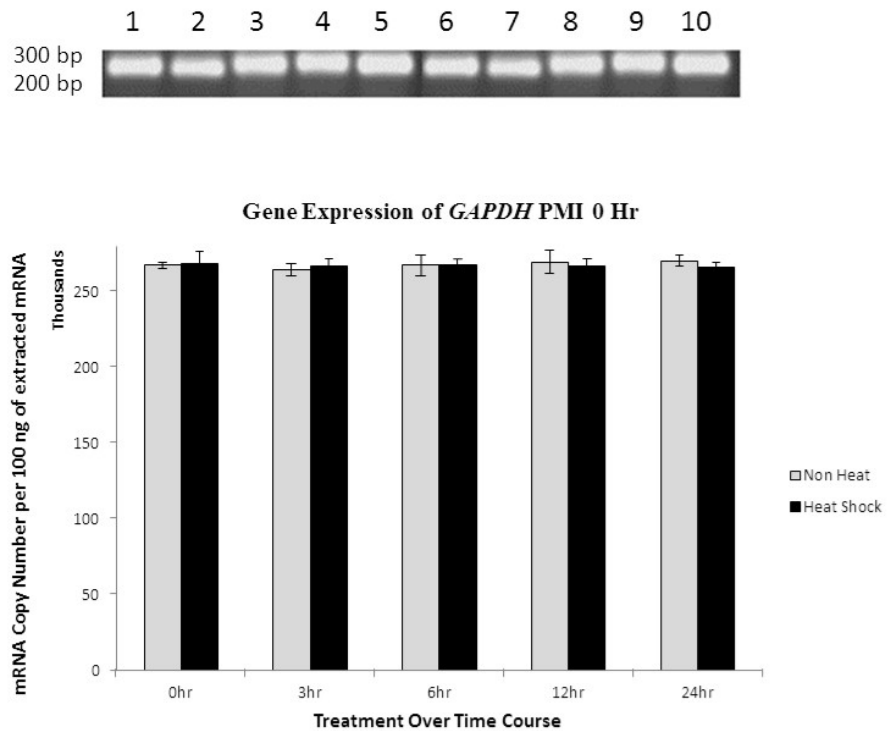


Figure 6.6 Transcription levels of *GAPDH* mRNA in non-heat shock and heat shocked rat brain tissue at 0 h PMI. (A) Agarose gel electrophoresis: Lane 1, 3, 5, 7 and 9 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. Lanes 2, 4, 6, 8 and 10 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. (B) Histogram showing *GAPDH* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6, 12 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars.

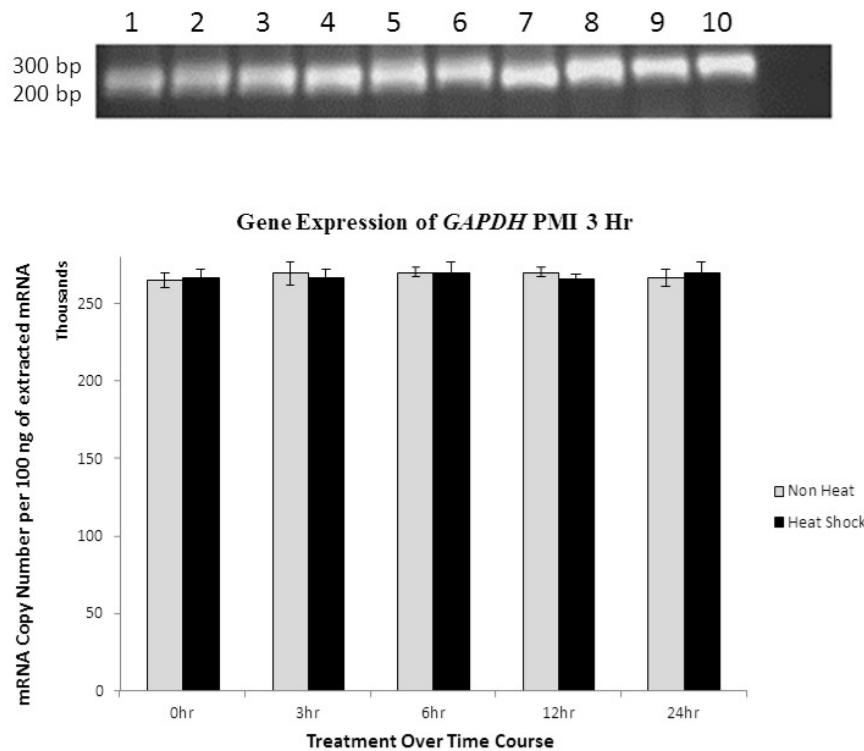


Figure 6.7 Transcription levels of *GAPDH* mRNA in non-heat shock and heat shocked rat brain tissue at 3 h PMI. (A) Agarose gel electrophoresis: Lane 1, 3, 5, 7 and 9 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. Lanes 2, 4, 6, 8 and 10 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. (B) Histogram showing *GAPDH* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6, 12 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars.

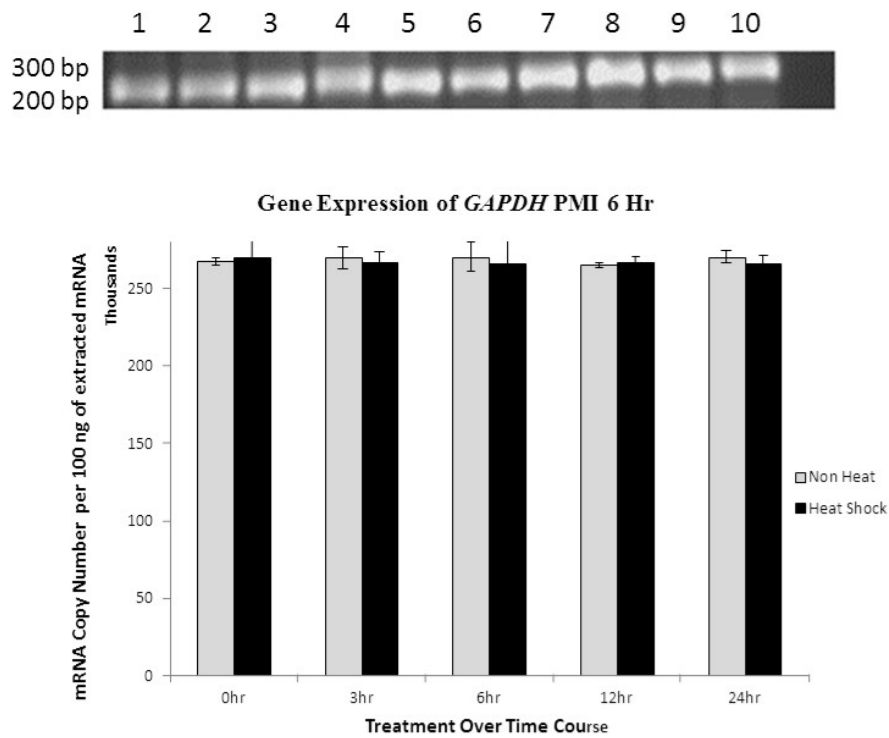


Figure 6.8 Transcription levels of *GAPDH* mRNA in non-heat shock and heat shocked rat brain tissue at 6 h PMI. (A) Agarose gel electrophoresis: Lane 1, 3, 5, 7 and 9 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. Lanes 2, 4, 6, 8 and 10 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. (B) Histogram showing *GAPDH* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6, 12 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars.

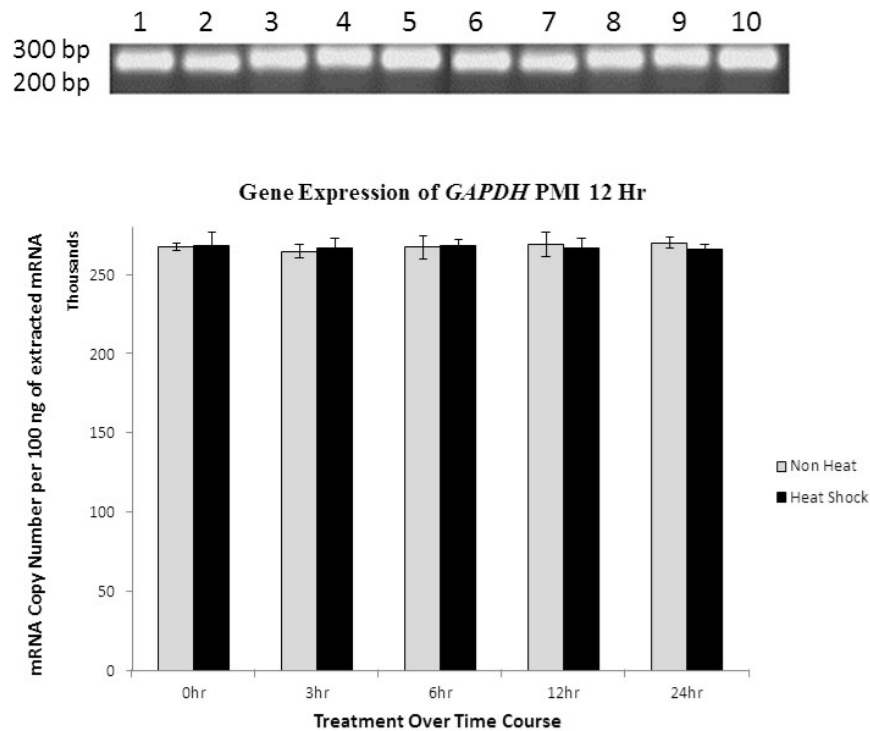


Figure 6.9 Transcription levels of *GAPDH* mRNA in non-heat shock and heat shocked rat brain tissue at 12 h PMI. (A) Agarose gel electrophoresis: Lane 1, 3, 5, 7 and 9 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. Lanes 2, 4, 6, 8 and 10 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. (B) Histogram showing *GAPDH* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6, 12 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars.

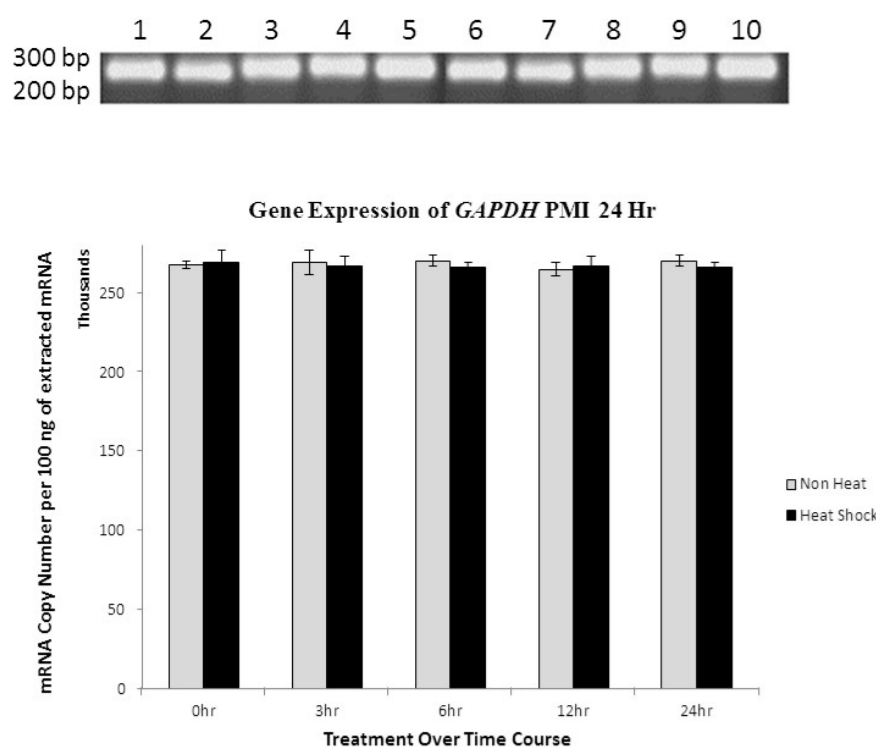


Figure 6.10 Transcription levels of *GAPDH* mRNA in non-heat shock and heat shocked rat brain tissue at 24 h PMI. (A) Agarose gel electrophoresis: Lane 1, 3, 5, 7 and 9 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. Lanes 2, 4, 6, 8 and 10 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6, 12 and 24 h respectively. (B) Histogram showing *GAPDH* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6, 12 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars.

6.2.2 Statistical Analysis

A three-way factorial mixed 5 (PMI: 0 h, 3 h, 6 h, 12 h, 24 h) x 2 (treatment: non-heat shock, heat shock) x 5 (time course: 0 h, 3 h, 6 h, 12 h, 24 h) ANOVA analysis of variance was performed to investigate differences of *HSPA* and *GAPDH* transcript copy numbers in rat brain tissue. The means and standard deviation are presented in Table 6.1 (*HSPA*) and Table 6.2 (*GAPDH*).

HSPA transcript copy numbers had a significant effect for time course [$F(4,17) = 443.06$, $P < .05$]; Wilks' Lambda = 0.010; partial eta squared = 0.990. *HSPA* transcript copy numbers had a significant effect between the interaction of: time course and PMI [$F(16,52.57) = 77.93$; $P < .05$], Wilks' Lambda = 0.000, partial eta squared = 0.914; time course and treatment [$F(4,17) = 47.30$; $P < .05$], Wilks' Lambda = 0.082, partial eta squared = 0.918; time course and PMI and treatment [$F(16,52.57) = 69.17$; $P < .05$], Wilks' Lambda = 0.000, partial eta squared = 0.906.

There was a statistically significant main effect in *HSPA* transcript copy numbers on the combined variables for PMI [$F(4,20) = 473.82$, $P < .05$]; partial eta squared (η_p^2) = 0.990; treatment [$F(1,20) = 431.83$, $P < .05$]; partial eta squared 0.956 and PMI and treatment [$F(4,20) = 45.76$, $P < .05$]; partial eta squared = 0.901.

When the results for the dependent variables were considered separately, there was a statistical significance in PMI at time course: 0 h [$F(4,20) = 208.28$, $P < .05$]; partial eta squared = .977; 3 h [$F(4,20) = 175.74$, $P < .05$]; partial eta squared = .972; 6 h [$F(4,20) = 271.82$, $P < .05$]; partial eta squared = .982; 12 h [$F(4,20) = 244.31$, $P < .05$]; partial eta squared = .980 and 24 h [$F(4,20) = 476.20$, $P < .05$]; partial eta squared = .855.

There was a statistical significance in treatment at time course: 0 h [$F(1,20) = 744.96$, $P < .05$]; partial eta squared = .973; 3 h [$F(1,20) = 124.37$, $P < .05$]; partial eta squared = .861; 12 h [$F(1,20) = 313.15$, $P < .05$]; partial eta squared = .940 and 24 h [$F(1,20) = 37.54$, $P < .05$]; partial eta squared = .652. There was a non-significant difference in treatment at time course 6 h [$F(1,20) = 2.70$, $P < .05$]; partial eta squared = .119.

There was a statistical significance in PMI and treatment at time course: 0 h [$F(4,20) = 166.73$, $P < .05$]; partial eta squared = .971; 3 h [$F(4,20) = 58.54$, $P < .05$]; partial eta squared = .921; 6 h [$F(4,20) = 75.95$, $P < .05$]; partial eta squared = .938; 12 h [$F(4,20) = 59.82$, $P < .05$]; partial eta squared = .923 and 24 h [$F(3,20) = 12.33$, $P < .05$]; partial eta squared = .712.

An independent-samples t-test was conducted to compare *HSPA* transcript copy numbers estimated marginal mean scores for non-heat shock and heat shock over time course by PMI (Figures 6.11 – 6.15).

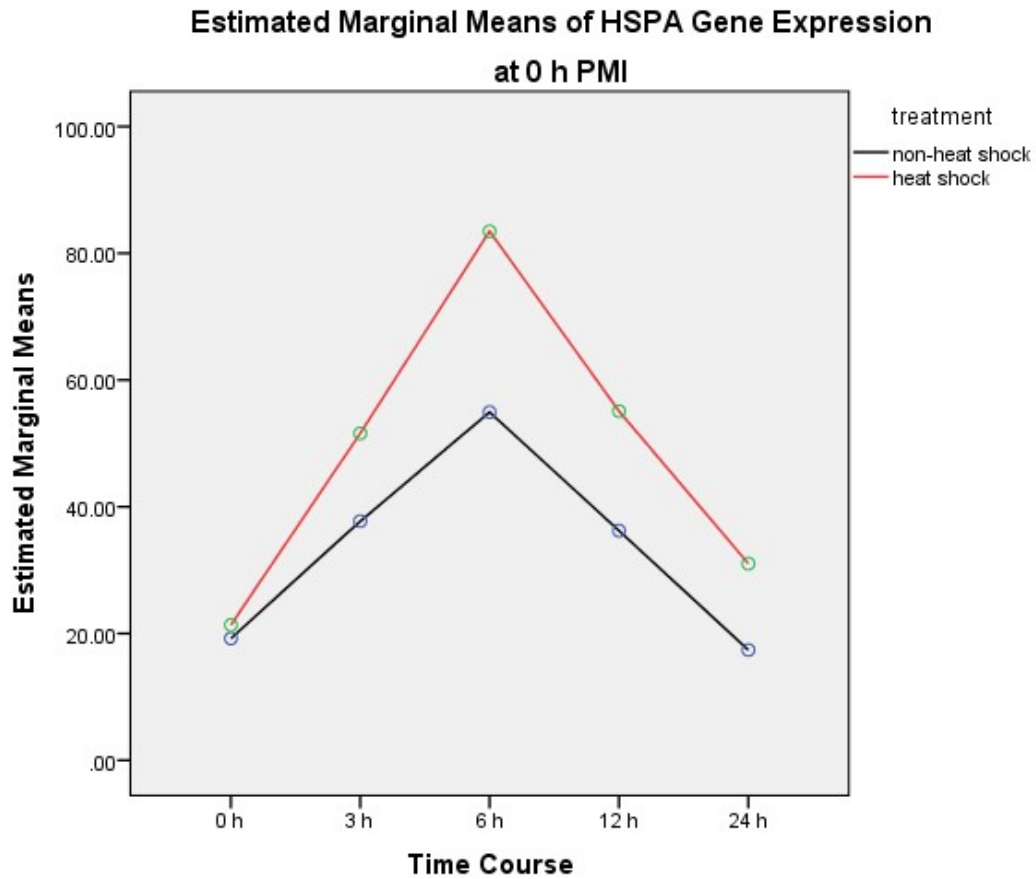


Figure 6.11 Estimated marginal means of *HSPA* transcript copy numbers at 0 h PMI. There was a non-significant difference in *HSPA* transcript copy numbers at 0 h PMI, 0 h time course between non-heat shock (MD = 19.21, SD = .922) and heat shock [MD 21.34, SD = 1.41; $t(4) = 2.181$, $P > .05$]. There was significantly lower *HSPA* transcript copy numbers at 0 h PMI, 3 h time course in non-heat shock (MD = 37.73, SD = .332) than heat shock [MD 51.57, SD = .986; $t(4) = 6.915$, $P = .002$]. There was significantly lower *HSPA* transcript copy numbers at 0 h PMI, 6 h time course in non-heat shock (MD = 54.94, SD = 4.47) than heat shock [MD 83.47, SD = 9.03; $t(4) = 4.903$, $P = .008$]. There was significantly lower *HSPA* transcript copy numbers at 0 h PMI, 12 h time course in non-heat shock (MD = 36.23, SD = .572) than heat shock [MD 55.10, SD = 1.58; $t(4) = 19.494$, $P < .05$]. There was significantly lower *HSPA* transcript copy numbers at 0 h PMI, 24 h time course in non-heat shock (MD = 17.40, SD = 2.79) than heat shock [MD 31.03, SD = 3.90; $t(4) = 4.921$, $P = .008$] (Table 6.1).

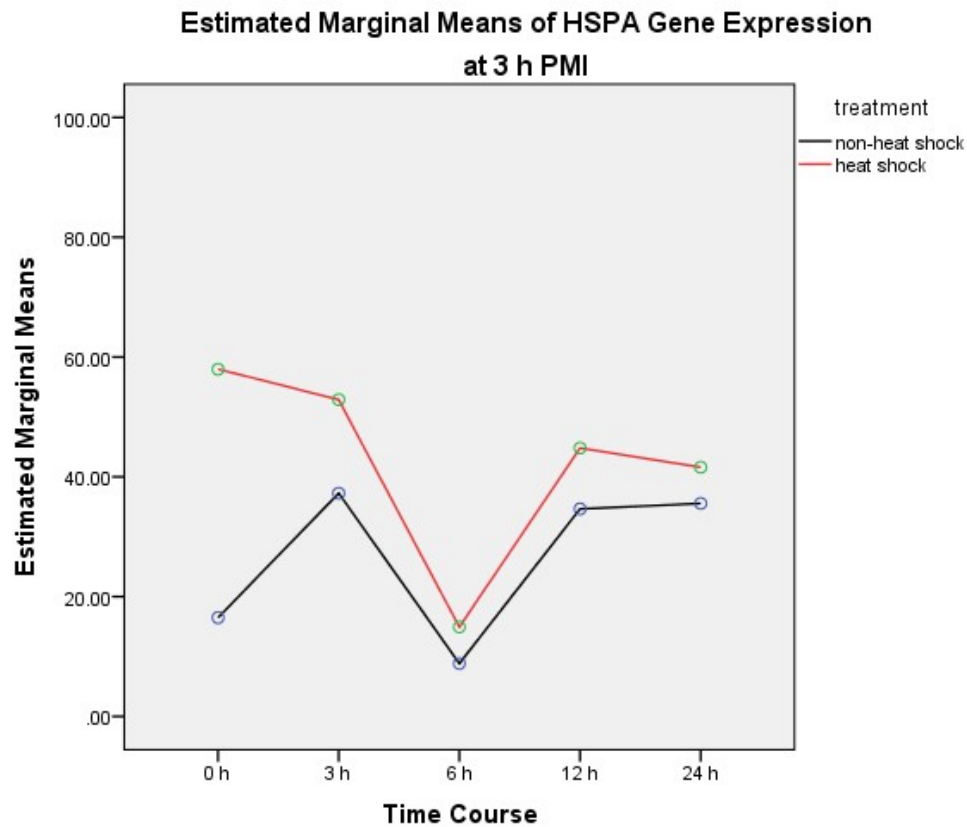


Figure 6.12 Estimated marginal means of *HSPA* transcript copy numbers at 3 h PMI. There was significantly lower *HSPA* transcript copy numbers at 3 h PMI, 0 h time course in non-heat shock (MD = 16.49, SD = .000) than heat shock [MD 57.96, SD = .000]. There was significantly lower *HSPA* transcript copy numbers at 3 h PMI, 3 h time course in non-heat shock (MD = 37.26, SD = .000) than heat shock [MD 52.89, SD = .000]. There was a non-significant difference in *HSPA* transcript copy numbers at 3 h PMI, 6 h time course between non-heat shock (MD = 8.83, SD = .000) and heat shock [MD 14.93, SD = .000]. There was a non-significant difference in *HSPA* transcript copy numbers at 3 h PMI, 12 h time course between non-heat shock (MD = 34.64, SD = 6.25) and heat shock [MD 44.80, SD = 5.08; $t(4) = 2.184$, $P > .05$]. There was a non-significant difference in *HSPA* transcript copy numbers at 3 h PMI, 24 h time course between non-heat shock (MD = 35.55, SD = 2.29) and heat shock [MD 41.59, SD = 7.51; $t(4) = 1.334$, $P > .05$] (Table 6.1).

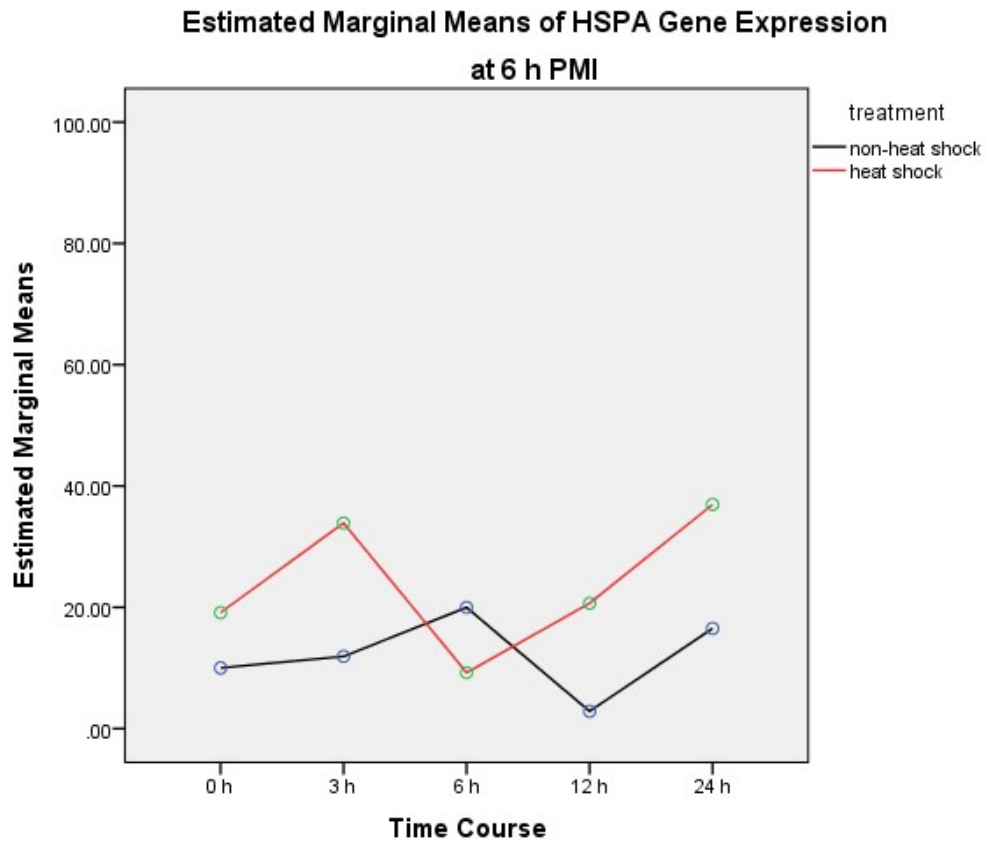


Figure 6.13 Estimated marginal means of *HSPA* transcript copy numbers at 6 h PMI. There was significantly lower *HSPA* transcript copy numbers at 6 h PMI, 0 h time course in non-heat shock (MD = 10.01, SD = 1.58) than heat shock [MD 19.13, SD = 2.07; $t(4) = 6.058$, $P = .004$]. There was significantly lower *HSPA* transcript copy numbers at 6 h PMI, 3 h time course in non-heat shock (MD = 11.91, SD = .943) than heat shock [MD 33.89, SD = 7.37; $t(4) = 5.124$, $P = .034$]. There was significantly higher *HSPA* transcript copy numbers at 6 h PMI, 6 h time course in non-heat shock (MD = 19.98, SD = 2.25) than heat shock [MD 9.22, SD = 2.95; $t(4) = 5.025$, $P = .007$]. There was significantly lower *HSPA* transcript copy numbers at 6 h PMI, 12 h time course in non-heat shock (MD = 2.86, SD = .496) than heat shock [MD 20.66, SD = .092; $t(4) = 81.08$, $P < .05$]. There was significantly lower *HSPA* transcript copy numbers at 6 h PMI, 24 h time course in non-heat shock (MD = 16.51, SD = 4.91) than heat shock [MD 36.96, SD = 6.14; $t(4) = 4.503$, $P = .011$]. (Table 6.1)

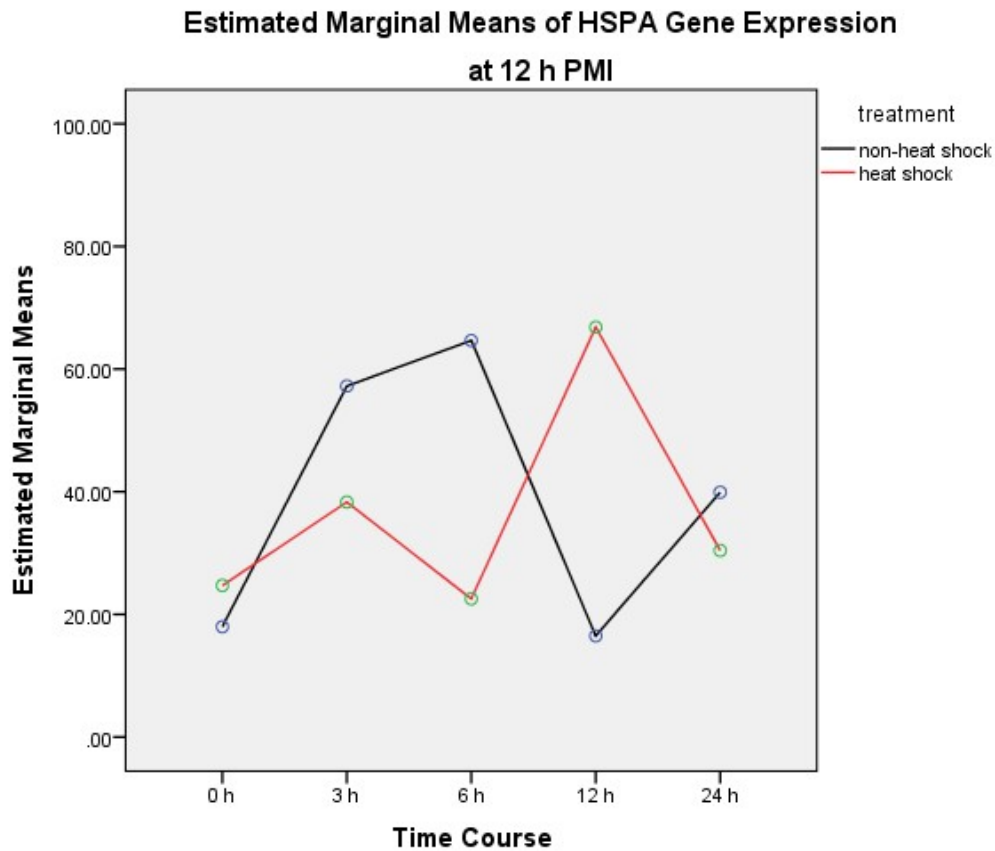


Figure 6.14 Estimated marginal means of *HSPA* transcript copy numbers at 12 h PMI. There was significantly lower *HSPA* transcript copy numbers at 12 h PMI, 0 h time course in non-heat shock (MD = 17.99, SD = .842) than heat shock [MD 24.71, SD = 1.24; $t(4) = 7.775$, $P = .001$]. There was significantly higher *HSPA* transcript copy numbers at 12 h PMI, 3 h time course in non-heat shock (MD = 57.23, SD = 1.01) than heat shock [MD 438.31, SD = .167; $t(4) = 31.998$, $P = .001$]. There was significantly higher *HSPA* transcript copy numbers at 12 h PMI, 6 h time course in non-heat shock (MD = 64.65, SD = .571) than heat shock [MD 22.54, SD = .782; $t(4) = 75.276$, $P < .05$]. There was significantly lower *HSPA* transcript copy numbers at 12 h PMI, 12 h time course in non-heat shock (MD = 16.49, SD = .000) than heat shock [MD 66.82, SD = .295; $t(4) = 296.06$, $P < .05$]. There was significantly higher *HSPA* transcript copy numbers at 12 h PMI, 24 h time course in non-heat shock (MD = 39.92, SD = 1.52) than heat shock [MD 30.41, SD = 2.77; $t(4) = 5.203$, $P = .007$] (Table 6.1).

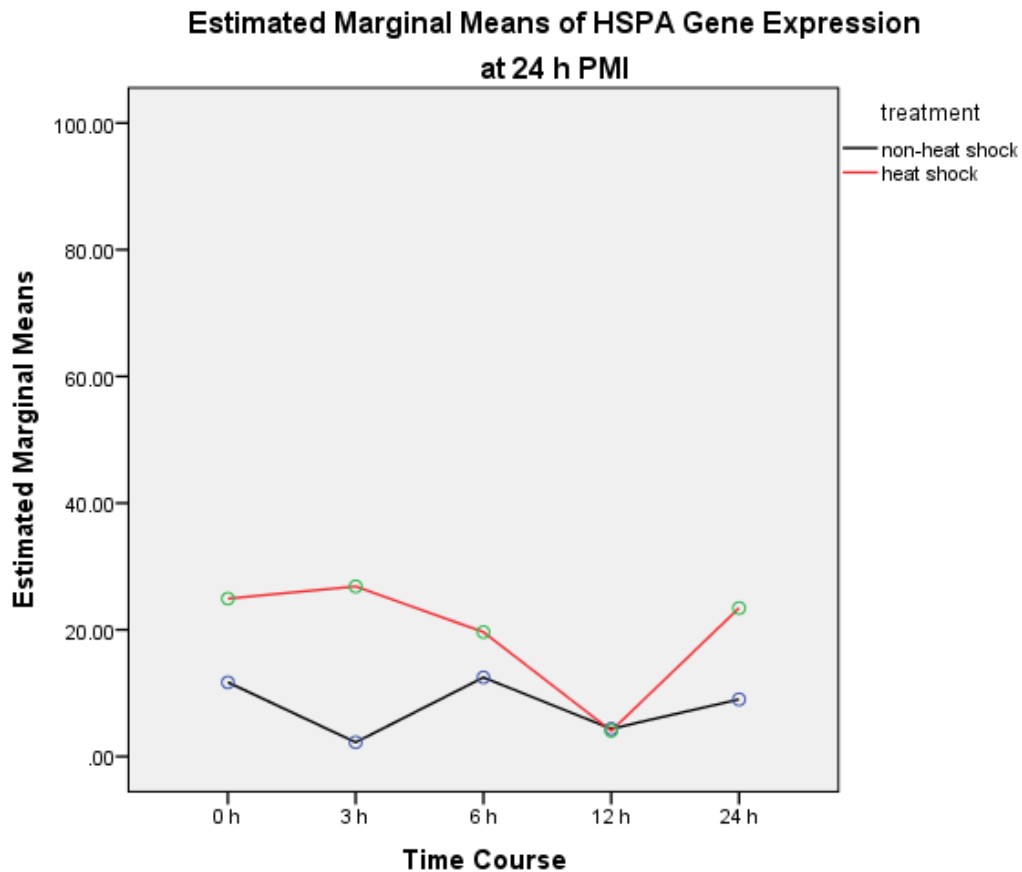


Figure 6.15 Estimated marginal means of *HSPA* transcript copy numbers at 24 h PMI. There was significantly lower *HSPA* transcript copy numbers at 24 h PMI, 0 h time course in non-heat shock (MD = 11.70, SD = 1.02) than heat shock [MD 24.93, SD = 2.98; $t(4) = 7.26$, $P = .002$]. There was significantly lower *HSPA* transcript copy numbers at 24 h PMI, 3 h time course in non-heat shock (MD = 2.24, SD = .000) than heat shock [MD 26.84, SD = 3.23; $t(4) = 13.183$, $P < .05$]. There was significantly lower *HSPA* transcript copy numbers at 24 h PMI, 6 h time course in non-heat shock (MD = 12.48, SD = 4.51) than heat shock [MD 19.64, SD = .010; $t(4) = 2.751$, $P < .05$]. There was a non-significant difference in *HSPA* transcript copy numbers at 24 h PMI, 12 h time course between non-heat shock (MD = 4.37, SD = 3.69) and heat shock [MD 4.05, SD = 2.86; $t(4) = .118$, $P > .05$]. There was significantly lower *HSPA* transcript copy numbers at 24 h PMI, 24 h time course in non-heat shock (MD = 9.02, SD = 1.95) than heat shock [MD 23.43, SD = 1.34; $t(4) = 10.56$, $P < .05$] (Table 6.1).

For *GAPDH* transcript copy numbers, had a non-significant effect for time course [F(4,17) = .270, P = .893]; Wilks' Lambda = 0.940; partial eta squared = 0.060. *GAPDH* transcript copy numbers had a non-significant effect between the interaction of: time course and PMI [F(16,52.57) = .280; P = .996], Wilks' Lambda = .779, partial eta squared = 0.061; time course and treatment [F(4,17) = 1.235; P = .333], Wilks' Lambda = 0.775, partial eta squared = 0.225; time course and PMI and treatment [F(16,52.57) = .763; P = .717], Wilks' Lambda = 0.528, partial eta squared = 0.147.

There was a non-significant effect in *GAPDH* transcript copy numbers on the combined variables for PMI [F(4,20) = .031, P = .998]; partial eta squared (η_p^2) = 0.006; treatment [F(1,20) = 1.044, P = .319]; partial eta squared 0.050 and PMI and treatment [F(4,20) = .055, P = .994]; partial eta squared = 0.011.

Post-hoc analyses using Tukey's HSD test indicated that all differences failed to reach significance.

When the results for the dependent variables were considered separately, there was a statistical non-significance in PMI at time course: 0 h [F(4,20) = .250, P = .906]; partial eta squared = .048; 3 h [F(4,20) = .293, P = .897]; partial eta squared = .055; 6 h [F(4,20) = .200, P = .935]; partial eta squared = .039; 12 h [F(4,20) = .365, P = .831]; partial eta squared = .068 and 24 h [F(4,20) = 0.000, P > .05]; partial eta squared = .000.

There was a statistical non-significance in treatment at time course: 0 h [F(1,20) = .783, P = .387]; partial eta squared = .038; 3 h [F(1,20) = .150, P = .703]; partial eta squared = .007; 6 h [F(1,20) = .769, P = .391]; partial eta squared = .037; 12 h [F(1,20) = .326, P = .574]; partial eta squared = .016 and 24 h [F(1,20) = 3.71, P = .068]; partial eta squared = .156.

There was a statistical non-significance in PMI and treatment at time course: 0 h [$F(1,20) = .006$, $P > .05$]; partial eta squared = .001; 3 h [$F(1,20) = .293$, $P = .879$]; partial eta squared = .055; 6 h [$F(1,20) = .392$, $P = .812$]; partial eta squared = .073; 12 h [$F(1,20) = .456$, $P = .767$]; partial eta squared = .083 and 24 h [$F(1,20) = .942$, $P = .460$]; partial eta squared = .159.

Overall inspection of the mean scores for *GAPDH* transcript copy numbers indicated no significant differences in PMI, time course and treatment (Table 6.2)

Table 6.1. Descriptive statistics showing the mean *HSPA* transcript copy numbers for untreated and treated brain tissue.

| PMI | Treatment | N | Time Course | | | | | | | | | |
|---------|-----------|---|-------------|-------------|--------|-------------|--------|-------------|---------|-------------|---------|-------------|
| | | | 0 Hour | | 3 Hour | | 6 Hour | | 12 Hour | | 24 Hour | |
| | | | Mean | STD Dev. | Mean | STD Dev. | Mean | STD Dev. | Mean | STD Dev. | Mean | STD Dev. |
| 0 Hour | Non | 3 | 19.21 | .923 | 37.73 | 3.32 | 54.94 | 4.47 | 36.23 | .572 | 17.40 | 2.79 |
| | Heat | 3 | 21.34 | 1.41 | 51.57 | .986 | 83.47 | 9.03 | 55.10 | 1.58 | 31.03 | 3.91 |
| 3 Hour | Non | 3 | 16.49 | .00 | 37.26 | .00 | 8.83 | .00 | 34.64 | 6.25 | 35.55 | 2.29 |
| | Heat | 3 | 57.96 | .00 | 52.89 | .00 | 14.93 | .00 | 44.80 | 5.08 | 41.59 | 7.51 |
| 6 Hour | Non | 3 | 10.01 | 1.58 | 11.91 | .943 | 19.98 | 2.25 | 2.86 | .497 | 16.51 | 4.91 |
| | Heat | 3 | 19.13 | 2.07 | 33.90 | 7.37 | 9.22 | 2.95 | 20.67 | .092 | 36.96 | 6.14 |
| 12 Hour | Non | 3 | 17.99 | .84 | 57.23 | 1.01 | 64.65 | .572 | 16.49 | .00 | 39.92 | 1.52 |
| | Heat | 3 | 24.71 | 1.24 | 38.31 | .167 | 22.54 | .782 | 66.82 | .295 | 30.41 | 2.78 |
| 24 Hour | Non | 3 | 11.70 | 1.03 | 2.24 | .00 | 12.48 | 4.51 | 4.37 | 3.69 | 9.02 | 1.95 |
| | Heat | 3 | 24.93 | 2.98 | 26.84 | 3.23 | 19.64 | .010 | 4.05 | 2.86 | 23.43 | 1.34 |

Table 6.2. Descriptive statistics showing the mean *GAPDH* transcript copy numbers for untreated and treated brain tissue.

| PMI | Treatment | N | Time Course | | | | | | | | | |
|---------|-----------|---|-------------|----------|-----------|----------|-----------|----------|-----------|----------|-----------|----------|
| | | | 0 Hour | | 3 Hour | | 6 Hour | | 12 Hour | | 24 Hour | |
| | | | Mean | STD Dev. | Mean | STD Dev. | Mean | STD Dev. | Mean | STD Dev. | Mean | STD Dev. |
| 0 Hour | Non | 3 | 267595.53 | 2348.43 | 264904.33 | 4036.84 | 267652.60 | 7175.04 | 269709.77 | 7719.68 | 270333.27 | 3140.67 |
| | Heat | 3 | 269034.67 | 8198.82 | 266945.90 | 5354.84 | 268281.23 | 3116.86 | 266945.90 | 5354.84 | 266239.67 | 2348.43 |
| 3 Hour | Non | 3 | 264904.33 | 4036.84 | 269709.77 | 7719.68 | 270333.27 | 3140.67 | 270333.27 | 3140.67 | 266949.90 | 5354.84 |
| | Heat | 3 | 266945.00 | 5353.81 | 266945.90 | 5354.84 | 269709.77 | 7719.68 | 266239.67 | 2348.43 | 269709.77 | 7719.68 |
| 6 Hour | Non | 3 | 267595.53 | 2348.43 | 269709.77 | 7719.68 | 270333.27 | 3140.67 | 264904.33 | 4036.84 | 270333.27 | 3140.67 |
| | Heat | 3 | 269709.772 | 7719.68 | 266945.90 | 5354.84 | 266239.67 | 2348.43 | 266945.90 | 5354.84 | 266239.67 | 2348.43 |
| 12 Hour | Non | 3 | 267595.43 | 2348.34 | 264904.33 | 4036.84 | 267652.60 | 7175.04 | 269709.77 | 7719.68 | 270333.27 | 3140.67 |
| | Heat | 3 | 269034.67 | 8198.82 | 266945.90 | 5354.84 | 268281.23 | 3116.86 | 266945.90 | 5354.84 | 266239.67 | 2348.43 |
| 24 Hour | Non | 3 | 267595.53 | 2348.43 | 269709.77 | 7719.68 | 270333.27 | 3140.67 | 264904.33 | 4036.84 | 270333.27 | 3140.67 |
| | Heat | 3 | 269709.77 | 7719.68 | 266945.90 | 5354.84 | 266239.67 | 2348.43 | 266945.90 | 5354.84 | 266239.67 | 2348.43 |

Table 6.3. Statistical data from Tukey's HSD test showing significance of *HSPA* transcript copy numbers between PMI. Any significant differences $P \leq 0.05$ are highlighted in bold.

| (I) PMI | J (PMI) | Mean Difference (I – J) | Std. Error | Sig. |
|-----------|-----------|-------------------------|-------------|-------------|
| 0 | 3 | 6.31 | .793 | .000 |
| | 6 | 22.69 | .793 | .000 |
| | 12 | 2.90 | .793 | .012 |
| | 24 | 26.93 | .793 | .000 |
| 3 | 6 | 16.38 | .793 | .000 |
| | 12 | 3.41 | .793 | .003 |
| | 24 | 20.62 | .793 | .000 |
| 6 | 12 | 19.79 | .793 | .000 |
| | 24 | 4.24 | .793 | .000 |
| 12 | 24 | 24.04 | .793 | .000 |

Post-hoc analyses using Tukey's HSD test indicated significantly lower *HSPA* transcript copy numbers in 0 h PMI than in: 3 h PMI (MD = 6.31, $P < .05$); 6 h PMI (MD = 22.69, $P < .05$); 12 h PMI (MD = 0.012, $P < .05$) and 24 h PMI (MD = 26.93, $P < .05$). There was significantly lower *HSPA* transcript copy numbers in 3 h PMI than in: 6 h PMI (MD = 16.38, $P < .05$); 12 h PMI (MD = 3.41, $P = .003$) and 24 h PMI (MD = 20.62, $P < .05$). There was significantly lower *HSPA* transcript copy numbers in 6 h PMI than in: 12 h PMI (MD = 19.79, $P < .05$) and 24 h PMI (MD = 4.24, $P < .05$). There was significantly lower *HSPA* transcript copy numbers in 3 h PMI than in: 6 h PMI (MD = 16.38, $P < .05$); 12 h PMI (MD = 3.41, $P = .003$) and 24 h PMI (MD = 20.62, $P < .05$). There was significantly lower *HSPA* transcript copy numbers in 12 h PMI than in 24 h PMI (MD = 24.04, $P < .05$) (Table 6.3).

6.2.3 Rat Brain tissue - thermotolerance

Expression of *HSPA* and *GAPDH* in thermotolerant tissue was evaluated using RT-PCR in rat post-mortem brain tissue, at post-mortem intervals of 0, 3, 6 and 24 h and at time course intervals of 0, 3, 6 and 24 h within each post-mortem interval for both control (non-heat shock) and mild heat shock treated samples. The primers and optimal temperatures used for the amplification of both genes are documented in Table 2.10 in section 2.5. All PCR experiments were carried out in triplicate for consistency and repeatability. For each gene analysed, a quantification graph was produced to confirm gene amplification. The resulting amplicons for *HSPA* and *GAPDH* were then analysed using agarose gel electrophoresis, represented by bands of 156 and 265 bp respectively.

At 3 h, 6 and 24 h post-mortem interval at 0, 3, 6 and 24 h time course, heat shocked brain tissue transcribed *HSPA* at a higher level compared to that of the non-heat shocked brain tissue (Figure 6.17, 6.18 and 6.19). At 0 h post-mortem interval *HSPA* transcribed higher in heat shocked brain tissue at time course 0, 3 and 6 h, but lower at the 24 h time course than non-heat shocked brain tissue (Figure 6.17).

GAPDH expression level remained relatively consistent throughout each PMI, time course and non-heat shock and heat shocked brain tissue confirming it as an ideal reference gene (Figures 6.20 – 6.23).

The mRNA copy number per 100 ng of extracted mRNA for both genes was calculated for each time course 0, 3, 6 and 24 h within each post-mortem interval 0, 3, 6 and 24 h for both non-heat shock and heat shock to monitor the gene expression level. mRNA copy numbers confirmed that at: 0 h post-mortem interval, *HSPA* was expressed at a higher level in heat shocked brain tissue at different time course intervals; with

approximately 13 copies after 0 h recovery, with approximately 19 copies after 3 h recovery and approximately 4 copies after 6 h recovery compare to non-heat shocked brain tissue at different time course intervals; with approximately 2 copies after 0 h recovery, approximately 2 copies after 3 h recovery and approximately 16 copies after 6 h recovery. Expression was lower in heat shocked brain tissue after 24 h recovery, approximately 19 copies compare to non-heat shocked brain tissue after 24 h recovery, approximately 19 copies (Figure 6.16).

At 3 h post-mortem interval, *HSPA* was expressed at a higher level in heat shocked brain tissue at different time course intervals; with approximately 37 copies after 0 h recovery, with approximately 20 copies after 3 h recovery, with approximately 59 copies after 6 h recovery and approximately 20 copies after 24 h recovery compared to non-heat shocked brain tissue at different time course intervals; with approximately 2 copies after 0 h recovery, with approximately 10 copies after 3 h recovery, with approximately 19 copies after 6 h recovery and approximately 19 copies after 24 h recovery (Figure 6.17).

At 6 h post-mortem interval, *HSPA* was expressed at a higher level in heat shocked brain tissue at different time course intervals; with approximately 130 copies after 0 h recovery, with approximately 167 copies after 3 h recovery, with approximately 66 copies after 6 h recovery and approximately 63 copies after 24 h recovery compare to non-heat shocked brain tissue at different time course intervals; with approximately 30 copies after 0 h recovery, with approximately 47 copies after 3 h recovery, with approximately 50 copies after 6 h recovery and approximately 46 copies after 24 h recovery (Figure 6.18).

At 24 h post-mortem interval, *HSPA* was expressed at a higher level in heat shocked brain tissue at different time course intervals; with approximately 51 copies after 0 h recovery, with approximately 59 copies after 3 h recovery, with approximately 66 copies after 6 h recovery and approximately 121 copies after 24 h recovery compared to non-heat shocked brain tissue at different time course intervals; with approximately 44 copies after 0 h recovery, with approximately 31 copies after 3 h recovery, with approximately 43 copies after 6 h recovery and approximate 59 copies after 24 h recovery (Figure 6.19).

GAPDH expression level remained relatively consistent throughout each PMI, time course in both non-heat shock and heat shocked brain tissue again confirming comparability of *HSPA* results (Figures 6.20 – 6.23).

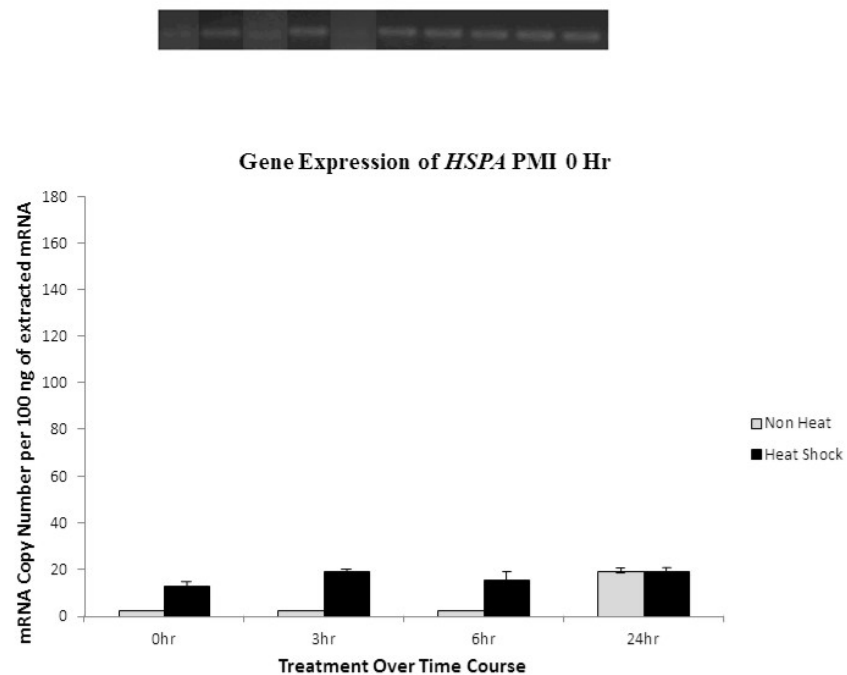


Figure 6.16 Transcription levels of *HSPA* mRNA in non-heat shock and heat shocked rat brain tissue at 0 h PMI. (A) Agarose gel electrophoresis: Lane 1, 3, 5 and 7 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6 and 24 h respectively. Lanes 2, 4, 6 and 8 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6 and 24 h respectively. (B) Histogram showing *HSPA* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars. At 0, 3 and 6 h non-heat shock no error bars are present as a 0 value standard deviation was calculated.

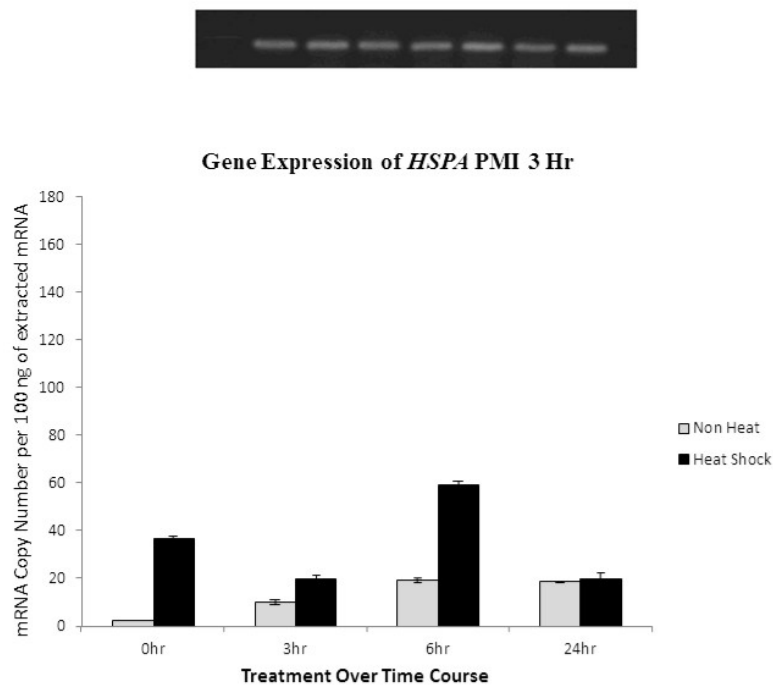


Figure 6.17 Transcription levels of *HSPA* mRNA in non-heat shock and heat shocked rat brain tissue at 3 h PMI. (A) Agarose gel electrophoresis: Lane 1, 3, 5 and 7 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6 and 24 h respectively. Lanes 2, 4, 6 and 8 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6 and 24 h respectively. (B) Histogram showing *HSPA* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars. At 0 h non-heat shock no error bars are present as a 0 value standard deviation was calculated.

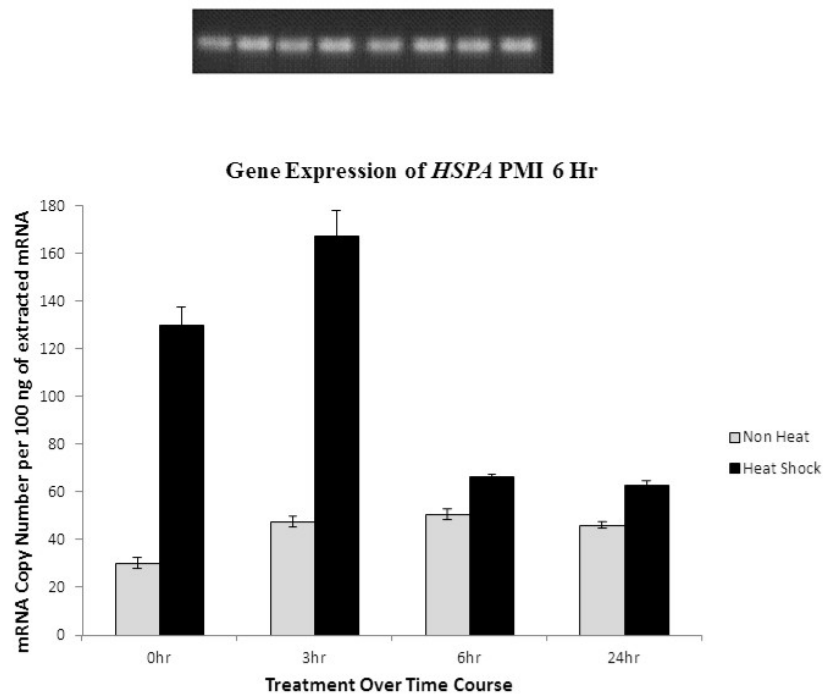


Figure 6.18 Transcription levels of *HSPA* mRNA in non-heat shock and heat shocked rat brain tissue at 6 h PMI. (A) Agarose gel electrophoresis: Lane 1, 3, 5 and 7 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6 and 24 h respectively. Lanes 2, 4, 6 and 8 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6 and 24 h respectively. (B) Histogram showing *HSPA* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars.

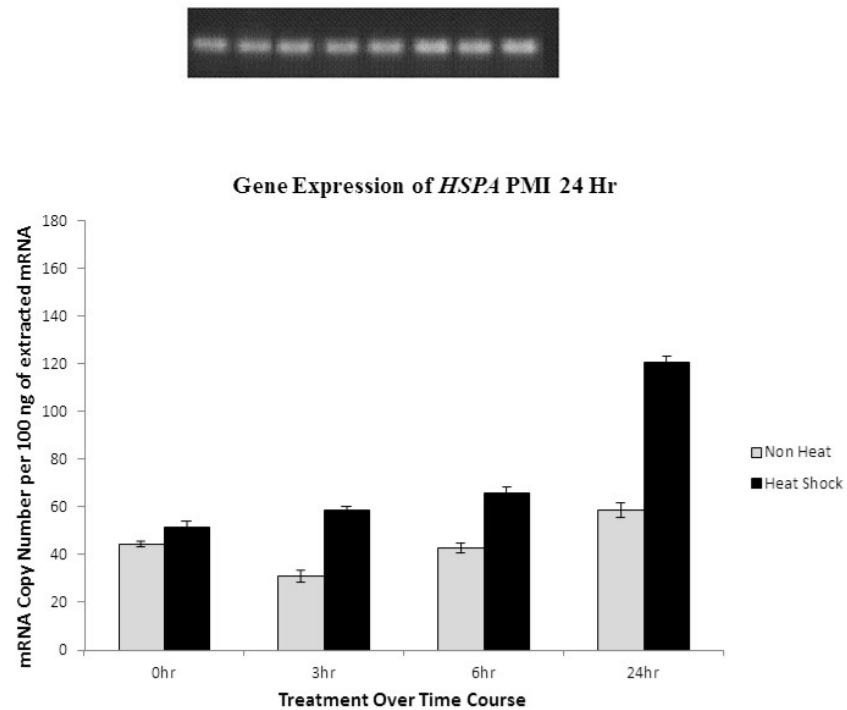


Figure 6.19 Transcription levels of *HSPA* mRNA in non-heat shock and heat shocked rat brain tissue at 24 h PMI. (A) Agarose gel electrophoresis: Lane 1, 3, 5 and 7 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6 and 24 h respectively. Lanes 2, 4, 6 and 8 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6 and 24 h respectively. (B) Histogram showing *HSPA* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars.

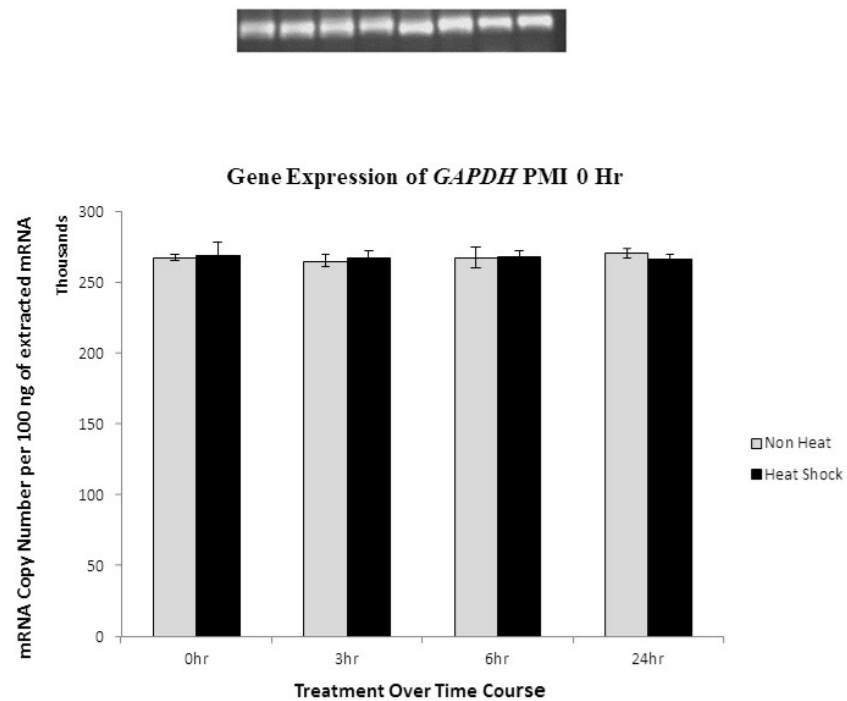


Figure 6.20 Transcription levels of *GAPDH* mRNA in non-heat shock and heat shocked rat brain tissue at 0 h PMI. (A) Agarose gel electrophoresis: Lane 1, 3, 5 and 7 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6 and 24 h respectively. Lanes 2, 4, 6 and 8 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6 and 24 h respectively. (B) Histogram showing *HSPA* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars.

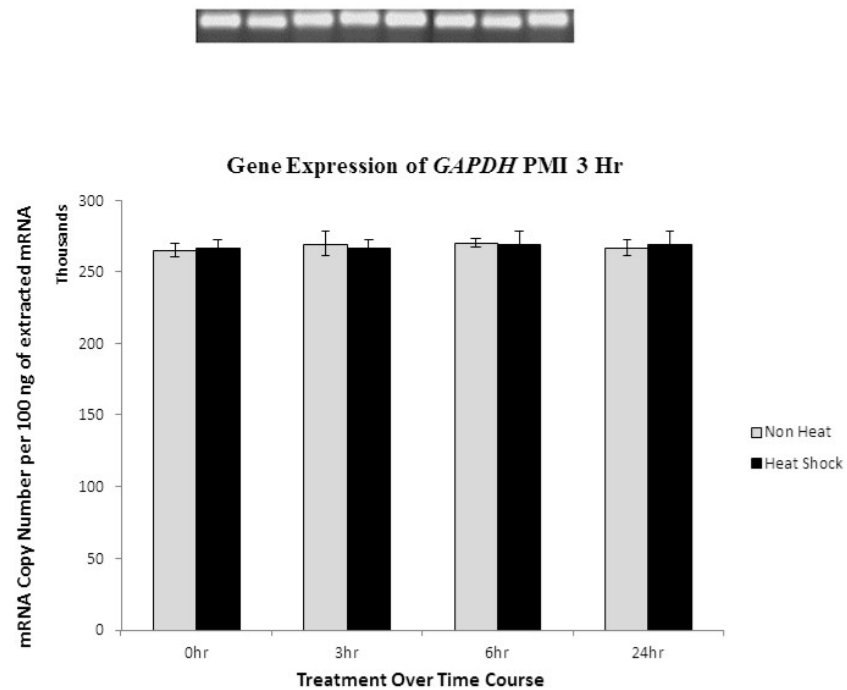


Figure 6.21 Transcription levels of *GAPDH* mRNA in non-heat shock and heat shocked rat brain tissue at 3 h PMI. (A) Agarose gel electrophoresis: Lane 1, 3, 5 and 7 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6 and 24 h respectively. Lanes 2, 4, 6 and 8 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6 and 24 h respectively. (B) Histogram showing *HSPA* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars.

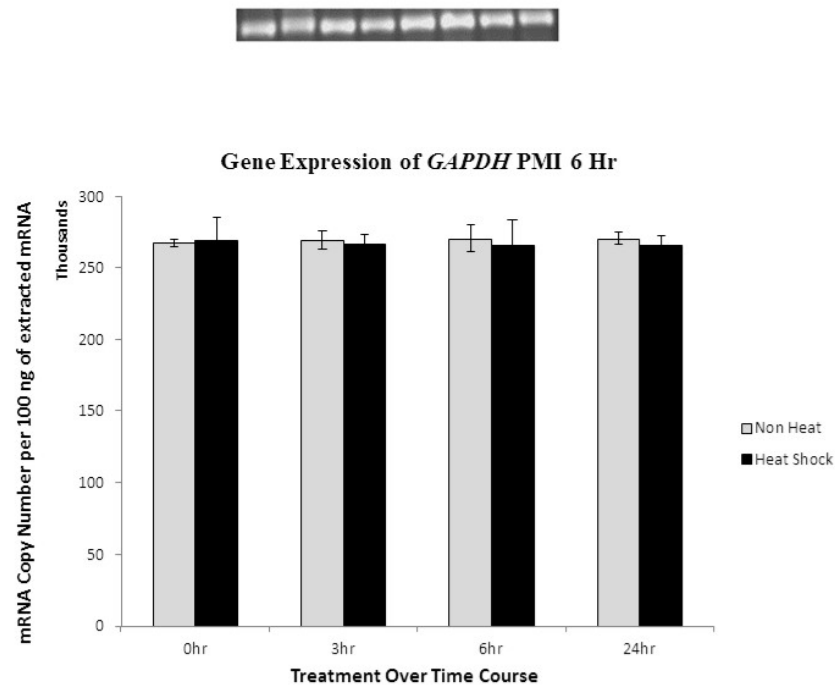


Figure 6.22 Transcription levels of *GAPDH* mRNA in non-heat shock and heat shocked rat brain tissue at 6 h PMI. (A) Agarose gel electrophoresis: Lane 1, 3, 5 and 7 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6 and 24 h respectively. Lanes 2, 4, 6 and 8 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6 and 24 h respectively. (B) Histogram showing *HSPA* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars.

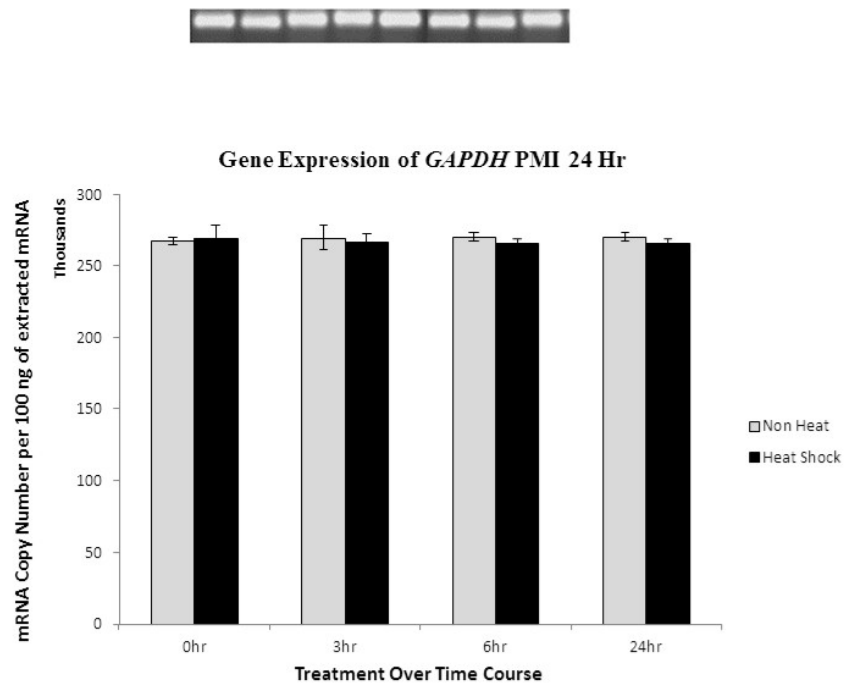


Figure 6.23 Transcription levels of *GAPDH* mRNA in non-heat shock and heat shocked rat brain tissue at 24 h PMI. (A) Agarose gel electrophoresis: Lane 1, 3, 5 and 7 represent amplicons from non-heat shocked rat brain tissue at time course intervals of 0, 3, 6 and 24 h respectively. Lanes 2, 4, 6 and 8 represent amplicons from heat shocked rat brain tissue at time course intervals of 0, 3, 6 and 24 h respectively. (B) Histogram showing *HSPA* mRNA copy numbers for non-heat shock and heat shocked brain tissue at 0, 3, 6 and 24 h time course intervals. Data values are the mean of three independent experiments, and the standard deviation from this mean is shown by the error bars.

6.2.4 Statistical Analysis

A three-way factorial mixed 4 (PMI: 0 h, 3 h, 6 h, 24 h) x 2 (treatment: non-heat shock, heat shock) x 4 (time course: 0 h, 3 h, 6 h, 24 h) ANOVA analysis of variance was performed to investigate differences of *HSPA* and *GAPDH* transcript copy numbers in rat brain tissue. The means and standard deviation are presented in Table 6.4 (*HSPA*) and Table 6.5 (*GAPDH*).

HSPA transcript copy numbers had a significant effect for time course [$F(3,14) = 108.67$, $P < .05$]; Wilks' Lambda = 0.041; partial eta squared = 0.959. *HSPA* transcript copy numbers had a significant effect between the interaction of: time course and PMI [$F(9,34.22) = 146.62$; $P < .05$], Wilks' Lambda = 0.000, partial eta squared = 0.949; time course and treatment [$F(3,14) = 50.74$; $P < .05$], Wilks' Lambda = 0.084, partial eta squared = 0.916; time course and PMI and treatment [$F(9,34.22) = 167.44$; $P < .05$], Wilks' Lambda = 0.000, partial eta squared = 0.954.

There was a statistically significant main effect in *HSPA* transcript copy numbers on the combined variables for PMI [$F(3,16) = 1809.72$, $P < .05$]; partial eta squared (η_p^2) = 0.997; treatment [$F(1,16) = 1982.83$, $P < .05$]; partial eta squared 0.992 and PMI and treatment [$F(3,16) = 265.01$, $P < .05$]; partial eta squared = 0.980.

When the results for the dependent variables were considered separately, there was a statistical significance in PMI at time course: 0 h [$F(3,16) = 680.86$, $P < .05$]; partial eta squared = .992; 3 h [$F(3,16) = 726.52$, $P < .05$]; partial eta squared = .993; 6 h [$F(3,16) = 759.73$, $P < .05$]; partial eta squared = .993 and 24 h [$F(3,16) = 2291.07$, $P < .05$]; partial eta squared = .998.

There was a statistical significance in treatment at time course: 0 h [$F(1,16) = 942.42$, $P < .05$]; partial eta squared = .983; 3 h [$F(1,16) = 695.39$, $P < .05$]; partial eta squared = .978; 6 h [$F(1,16) = 817.26$, $P < .05$]; partial eta squared = .981 and 24 h [$F(1,16) = 789.92$, $P < .05$]; partial eta squared = .980.

There was a statistical significance in PMI and treatment at time course: 0 h [$F(3,16) = 299.93$, $P < .05$]; partial eta squared = .983; 3 h [$F(3,16) = 241.46$, $P < .05$]; partial eta squared = .978; 6 h [$F(3,16) = 55.22$, $P < .05$]; partial eta squared = .912 and 24 h [$F(3,16) = 424.45$, $P < .05$]; partial eta squared = .988.

An independent-samples t-test was conducted to compare *HSPA* transcript copy numbers estimated marginal mean scores for non-heat shock and heat shock over time course by PMI (Figures 6.24 – 6.27).

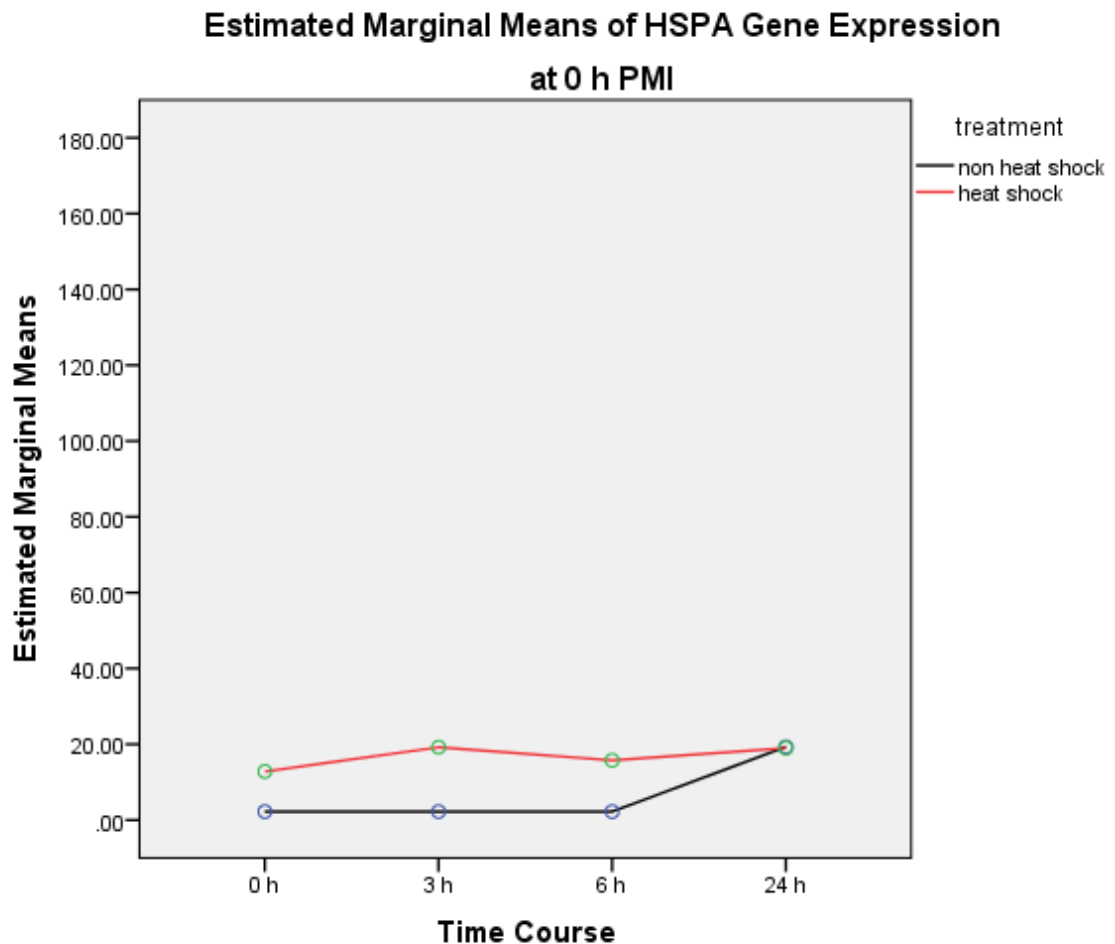


Figure 6.24 Estimated marginal means of *HSPA* transcript copy numbers at 0 h PMI. There was significantly lower in *HSPA* transcript copy numbers at 0 h PMI, 0 h time course in non-heat shock (MD = 2.24, SD = .00) than heat shock [MD 12.80, SD = 1.98; $t(4) = 9.258$, $P = .001$]. There was significantly lower *HSPA* transcript copy numbers at 0 h PMI, 3 h time course in non-heat shock (MD = 2.24, SD = .00) than heat shock [MD 19.20, SD = 0.526; $t(4) = 55.90$, $P < .05$]. There was significantly lower *HSPA* transcript copy numbers at 0 h PMI, 6 h time course in non-heat shock (MD = 2.24, SD = .00) than heat shock [MD 15.75, SD = 3.67; $t(4) = 6.38$, $P = .024$]. There was a non-significant difference in *HSPA* transcript copy numbers at 0 h PMI, 24 h time course between non-heat shock (MD = 19.31, SD = 0.84) and heat shock [MD 18.99, SD = 1.23; $t(4) = 0.372$, $P = .729$] (Table 6.4).

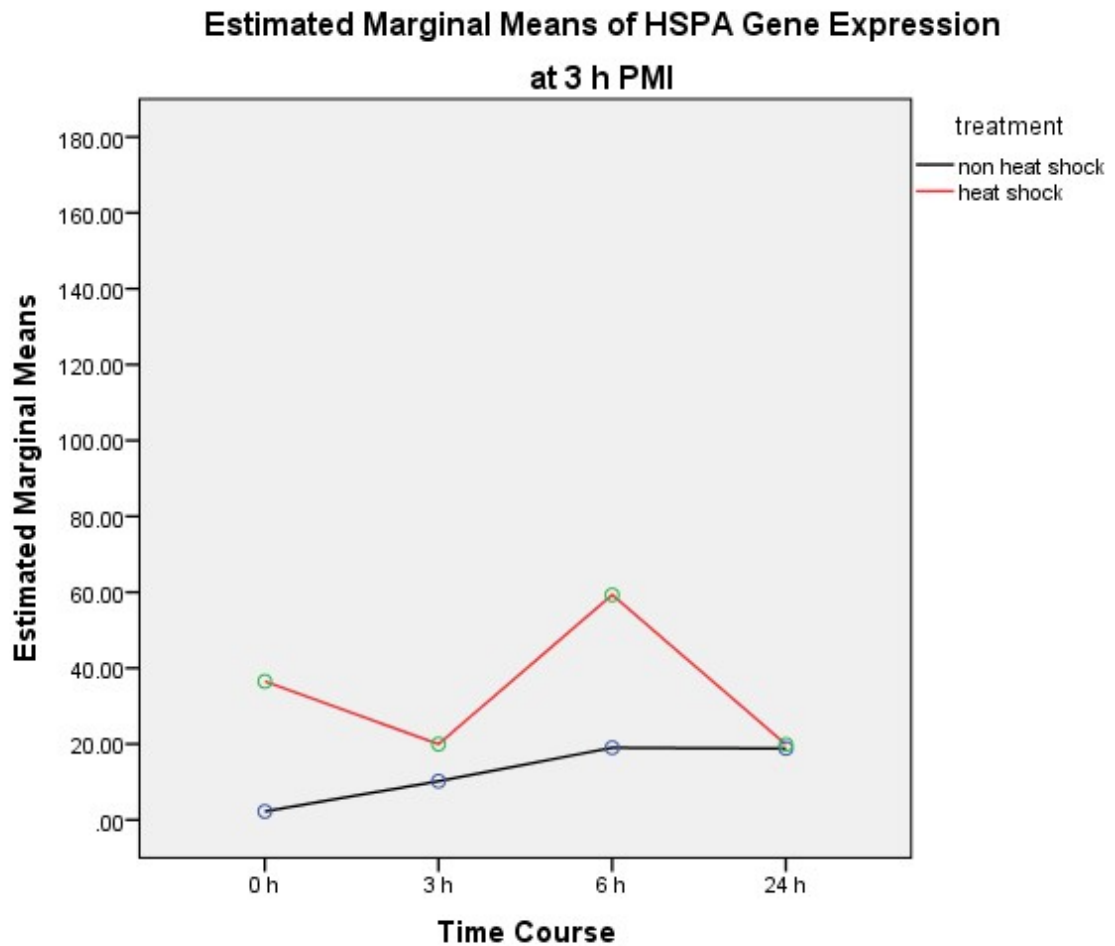


Figure 6.25 Estimated marginal means of *HSPA* transcript copy numbers at 3 h PMI. There was significantly lower *HSPA* transcript copy numbers at 3 h PMI, 0 h time course in non-heat shock (MD = 2.24, SD = .00) than heat shock [MD 36.51, SD = .647; $t(4) = 91.80$, $P < .05$]. There was significantly lower *HSPA* transcript copy numbers at 3 h PMI, 3 h time course in non-heat shock (MD = 10.21, SD = 1.03) than heat shock [MD 19.97, SD = 1.07; $t(4) = 11.40$, $P < .05$]. There was significantly lower *HSPA* transcript copy numbers at 3 h PMI, 6 h time course in non-heat shock (MD = 19.01, SD = .811) than heat shock [MD 59.30, SD = .785; $t(4) = 61.80$, $P < .05$]. There was a non-significant difference in *HSPA* transcript copy numbers at 3 h PMI, 24 h time course between non-heat shock (MD = 18.82, SD = .438) and heat shock [MD 19.90, SD = 1.70; $t(4) = 1.06$, $P = .388$] (Table 6.4).

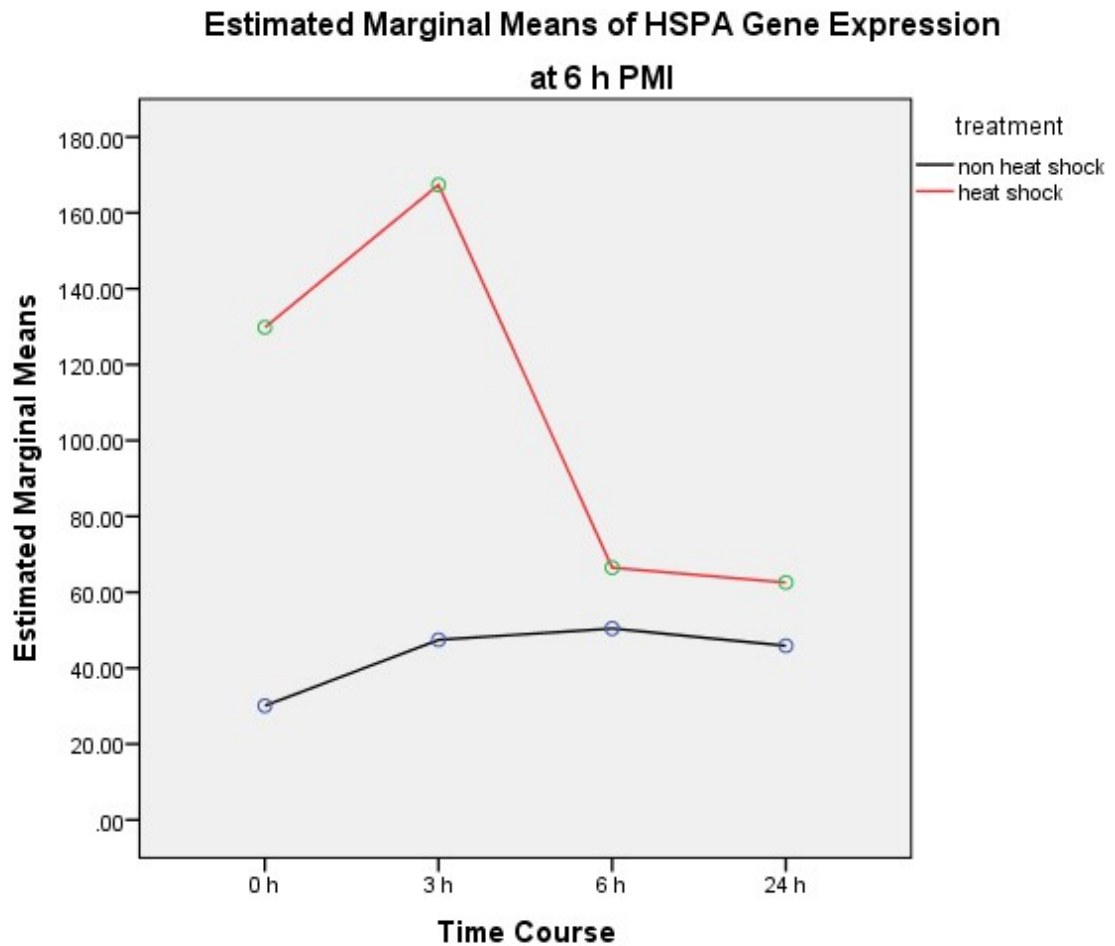


Figure 6.26 Estimated marginal means of *HSPA* transcript copy numbers at 6 h PMI. There was significantly lower *HSPA* transcript copy numbers at 6 h PMI, 0 h time course in non-heat shock (MD = 30.08, SD = 2.30) than heat shock [MD 129.81, SD = 7.59; $t(4) = 21.79$, $P < .05$]. There was significantly lower *HSPA* transcript copy numbers at 6 h PMI, 3 h time course in non-heat shock (MD = 47.45, SD = 2.07) than heat shock [MD 167.39, SD = 10.83; $t(4) = 18.84$, $P = .002$]. There was significantly lower *HSPA* transcript copy numbers at 6 h PMI, 6 h time course in non-heat shock (MD = 50.44, SD = 2.19) than heat shock [MD 66.48, SD = .878; $t(4) = 11.79$, $P < .05$]. There was significantly lower *HSPA* transcript copy numbers at 6 h PMI, 24 h time course in non-heat shock (MD = 45.89, SD = 1.01) than heat shock [MD 62.57, SD = 1.91; $t(4) = 13.41$, $P = .001$] (Table 6.4).

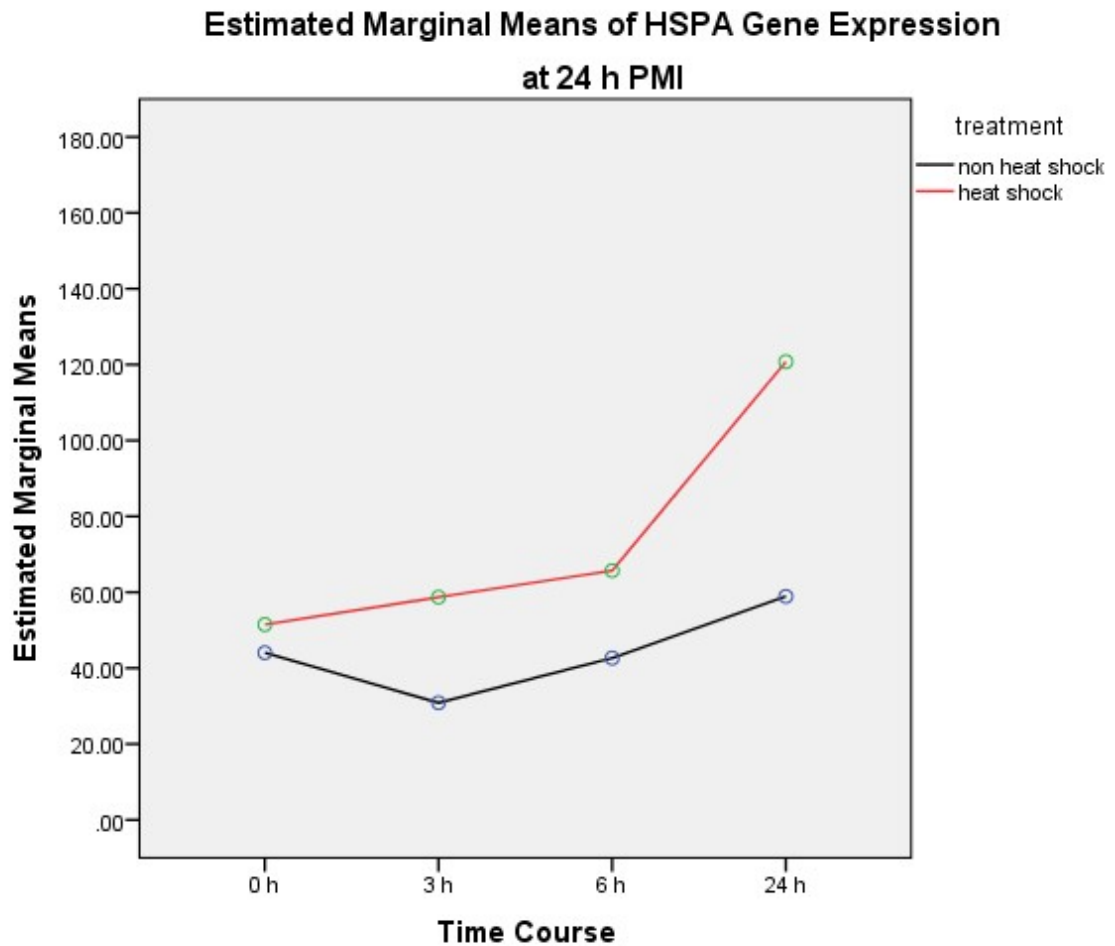


Figure 6.27 Estimated marginal means of *HSPA* transcript copy numbers at 24 h PMI. There was significantly lower *HSPA* transcript copy numbers at 24 h PMI, 0 h time course in non-heat shock (MD = 44.06, SD = 0.583) than heat shock [MD 51.47, SD = 2.45; $t(4) = 5.093$, $P = .029$]. There was significantly lower *HSPA* transcript copy numbers at 24 h PMI, 3 h time course in non-heat shock (MD = 30.86, SD = 2.49) than heat shock [MD 58.71, SD = 1.03; $t(4) = 17.88$, $P < .05$]. There was significantly lower *HSPA* transcript copy numbers at 24 h PMI, 6 h time course in non-heat shock (MD = 42.65, SD = 1.85) than heat shock [MD 65.68, SD = 2.82; $t(4) = 11.82$, $P = .001$]. There was significantly lower *HSPA* transcript copy numbers at 24 h PMI, 24 h time course in non-heat shock (MD = 58.90, SD = 3.06) than heat shock [MD 120.73, SD = 2.14; $t(4) = 28.71$, $P < .05$] (Table 6.4).

For *GAPDH* transcript copy numbers had a non-significant effect for time course [$F(3,14) = .163$, $P = .920$]; Wilks' Lambda = 0.966; partial eta squared = 0.034. *GAPDH* gene expression had a non-significant effect between the interaction of: time course and PMI [$F(9,34.22) = .367$; $P = .943$], Wilks' Lambda = .799, partial eta squared = 0.072; time course and treatment [$F(3,14) = 1.020$; $P = .413$], Wilks' Lambda = 0.821, partial eta squared = 0.179; time course and PMI and treatment [$F(9,34.22) = .766$; $P = .717$], Wilks' Lambda = 0.648, partial eta squared = 0.138.

There was a non-significant effect in *GAPDH* transcript copy numbers on the combined variables for PMI [$F(3,16) = .085$, $P = .967$]; partial eta squared (η_p^2) = 0.016; treatment [$F(1,16) = 0.672$, $P = .424$]; partial eta squared 0.040 and PMI and treatment [$F(3,16) = .313$, $P = .815$]; partial eta squared = 0.056.

Post-hoc analyses using Tukey's HSD test indicated that all differences failed to reach significance.

When the results for the dependent variables were considered separately, there was a statistical non-significance in PMI at time course: 0 h [$F(3,16) = .336$, $P = .800$]; partial eta squared = .059; 3 h [$F(3,16) = .224$, $P = .879$]; partial eta squared = .040; 6 h [$F(3,16) = .259$, $P = .854$]; partial eta squared = .046 and 24 h [$F(3,16) = 0.00$, $P > .05$]; partial eta squared = .000.

There was a statistical non-significance in treatment at time course: 0 h [$F(1,16) = .719$, $P = .409$]; partial eta squared = .043; 3 h [$F(1,16) = .378$, $P = .547$]; partial eta squared = .023; 6 h [$F(1,16) = 1.244$, $P = .281$]; partial eta squared = .072 and 24 h [$F(1,16) = 2.022$, $P = .174$]; partial eta squared = .112.

There was a statistical non-significance in PMI and treatment at time course: 0 h [$F(3,16) = .005$, $P = > .999$]; partial eta squared = .001; 3 h [$F(3,16) = .224$, $P = .879$];

partial eta squared.040; 6 h [F(3,16)435, P.731]; partial eta squared.075 and
24 h [F(3,16)1.050, P.398]; partial eta squared.164.

Table 6.4. Descriptive statistics showing the mean *HSPA* transcript copy numbers for untreated and treated brain tissue.

| PMI | Treatment | N | Time Course | | | | | | | |
|---------|-----------|---|-------------|-------------|--------|-------------|--------|-------------|---------|-------------|
| | | | 0 Hour | | 3 Hour | | 6 Hour | | 24 Hour | |
| | | | Mean | STD Dev. | Mean | STD Dev. | Mean | STD Dev. | Mean | STD Dev. |
| 0 Hour | Non | 3 | 2.24 | .00 | 2.24 | .00 | 2.24 | .00 | 19.31 | .837 |
| | Heat | 3 | 12.80 | 1.98 | 19.20 | .526 | 15.75 | 3.67 | 18.99 | 1.24 |
| 3 Hour | Non | 3 | 2.24 | .00 | 10.21 | 1.03 | 19.02 | .811 | 18.82 | .437 |
| | Heat | 3 | 36.51 | .647 | 19.97 | 1.07 | 59.30 | .785 | 19.90 | 1.70 |
| 6 Hour | Non | 3 | 30.08 | 2.30 | 47.45 | 2.07 | 50.44 | 2.19 | 45.89 | 1.01 |
| | Heat | 3 | 129.81 | 7.59 | 167.39 | 10.83 | 66.48 | .878 | 62.57 | 1.91 |
| 24 Hour | Non | 3 | 44.06 | .583 | 30.86 | 2.49 | 42.65 | 1.85 | 58.90 | 3.06 |
| | Heat | 3 | 51.47 | 2.45 | 58.71 | 1.03 | 65.68 | 2.82 | 120.76 | 2.14 |

Table 6.5. Descriptive statistics showing the mean *GAPDH* transcript copy numbers for untreated and treated brain tissue.

| PMI | Treatment | N | Time Course | | | | | | | |
|---------|-----------|---|-------------|----------|-----------|----------|-----------|----------|-----------|----------|
| | | | 0 Hour | | 3 Hour | | 6 Hour | | 24 Hour | |
| | | | Mean | STD Dev. | Mean | STD Dev. | Mean | STD Dev. | Mean | STD Dev. |
| 0 Hour | Non | 3 | 267595.53 | 2348.43 | 264904.33 | 4036.84 | 267652.60 | 7175.04 | 270333.27 | 3140.67 |
| | Heat | 3 | 269034.67 | 8198.82 | 266945.90 | 5354.84 | 268281.23 | 3116.86 | 266239.67 | 2348.43 |
| 3 Hour | Non | 3 | 264904.33 | 4036.84 | 269709.77 | 7719.68 | 270333.27 | 3140.67 | 266945.90 | 5354.84 |
| | Heat | 3 | 266945.00 | 5353.81 | 266945.90 | 5354.84 | 269709.77 | 7719.68 | 269709.77 | 7719.68 |
| 6 Hour | Non | 3 | 267595.53 | 2348.43 | 269709.77 | 7719.68 | 270333.27 | 3140.67 | 270333.27 | 3140.67 |
| | Heat | 3 | 269709.77 | 7719.68 | 266945.90 | 5354.84 | 266239.67 | 2348.43 | 266239.67 | 2348.43 |
| 24 Hour | Non | 3 | 267595.53 | 2348.43 | 269709.77 | 7719.68 | 270333.27 | 3140.67 | 270333.27 | 3140.67 |
| | Heat | 3 | 269709.77 | 7719.68 | 266945.90 | 5354.84 | 266239.67 | 2348.43 | 266239.67 | 2348.43 |

Overall inspection of the mean scores for *GAPDH* transcript copy numbers indicated no significant differences in PMI, time course and treatment (Table 6.5)

Table 6.6. Statistical data from Tukey's HSD test showing significance of *HSPA* transcript copy numbers between PMI. Any significant differences $P \leq 0.05$ are highlighted in bold.

| (I) PMI | J (PMI) | Mean Difference (I – J) | Std. Error | Sig. |
|----------|-----------|-------------------------|-------------|-------------|
| 0 | 3 | 11.65 | .990 | .000 |
| | 6 | 63.42 | .990 | .000 |
| | 24 | 47.54 | .990 | .000 |
| 3 | 6 | 51.77 | .990 | .000 |
| | 24 | 35.89 | .990 | .000 |
| 6 | 24 | 35.89 | .990 | .000 |

Post-hoc analyses using Tukey's HSD test indicated significantly lower *HSPA* transcript copy numbers in 0 h PMI than in: 3 h PMI (MD = 11.65, $P < .05$); 6 h PMI (MD = 63.42, $P < .05$) and 24 h PMI (MD = 47.54, $P < .05$). There was significantly lower *HSPA* transcript copy numbers in 3 h PMI than in: 6 h PMI (MD = 51.77, $P < .05$) and 24 h PMI (MD = 35.90, $P < .05$). There was significantly lower *HSPA* transcript copy numbers in 6 h PMI than in: 24 h PMI (MD = 15.88, $P < .05$) (Table 6.6).

6.3 Immunofluorescence

Under normal conditions HSPA protein in cells is localized in the cytoplasm, however under conditions of stress HSPA migrates to the nucleus. Immunofluorescence detection staining was carried out utilising a monoclonal primary HSPA antibody to identify the presence and localization of HSPA protein in post-mortem rat brain tissue for any comparable differences between control (unstressed) and under heat shock (stressed) conditions.

The tissue sections were initially fixed in freshly made 4% Paraformaldehyde (w/v) in PBS (0.1 M) for 15 min at room temperature, following which the excess paraformaldehyde was removed. The fixed tissue sections were permeabilized using Trypsin (0.025%) in aqueous calcium chloride (CaCl_2) (0.1%, pH 7.8) and incubated for 45 min at 37°C, then washed three times in warm PBS (0.1M). The sections were then incubated in blocking solution (0.1% PBS, 0.5% Tween 20, 0.1% Goat serum) for 30 min at room temperature, followed by overnight incubation at 4°C in the primary antibody for HSPA (Anti-Hsp70 antibody [BRM-22], Abcam,UK, dilution 1:200) to allow the antibody to bind and for permitted saturation to take place. After overnight incubation, the sections were washed with PBS three times for 5 min and incubated in a light sensitive Anti-mouse IgG FITC (Fluorescein Isothiocyanate) conjugated secondary detection antibody (Goat polyclonal Secondary Antibody to Mouse IgG - H&L FITC, Abcam, UK, dilution 1:200) diluted in blocking solution for 1 h at room temperature. The secondary antibody was removed followed by three consecutive washes with warm PBS (0.1 M). The sections were mounted under a cover slip using VECTASHIELD PI (0.01 M) (Propidium Iodide, Vector, USA) mounting medium. The tissue sections were then examined and images recorded using an Axiovert 200 LSM 510 laser scanning confocal microscope (Carl Zeiss, USA). Negative control cells from each sample

encountered identical preparations for immunofluorescence staining, except that the primary antibody was omitted.

HSPA antigens detected using an Anti-mouse IgG FITC conjugated secondary antibody were identified in the non-heat shock brain tissue, at 0 h post-mortem interval: in the cytoplasm at 0, 3 and 24 h time course, predominantly in the cytoplasm with a little fluorescence emitted from the nucleus at 6 h time course and in both cytoplasm and nucleus at 12 h time course (Figure 6.28). At 3 h post-mortem interval fluorescence was emitted: in the cytoplasm at 0 h time course and predominantly in the cytoplasm with a little fluorescence emitted from the nucleus at 3, 6, 12 and 24 h time course (Figure 6.29). At 6 h post-mortem interval fluorescence was emitted: in both the cytoplasm and nucleus at 0, 3 and 6 h time course and predominantly in the cytoplasm with a little fluorescence emitted from the nucleus at 12 and 24 h time course (Figure 6.30). At 12 h post-mortem interval fluorescence was emitted: in both the cytoplasm and nucleus at 0 h time course and predominantly in the cytoplasm with a little fluorescence emitted from the nucleus at 3, 6, 12 and 24 h time course (Figure 6.31). At 24 h post-mortem interval fluorescence was emitted predominantly in the cytoplasm with a little fluorescence emitted from the nucleus at 0, 3, 6, 12 and 24 h time course (Figure 6.32).

In heat shocked brain tissue HSPA protein was detected in both the cytoplasm and the nucleus for each time course 0, 3, 6, 12 and 24 h for each post-mortem interval 0, 3, 6, 12 and 24 h (Figures 6.33 – 6.37).

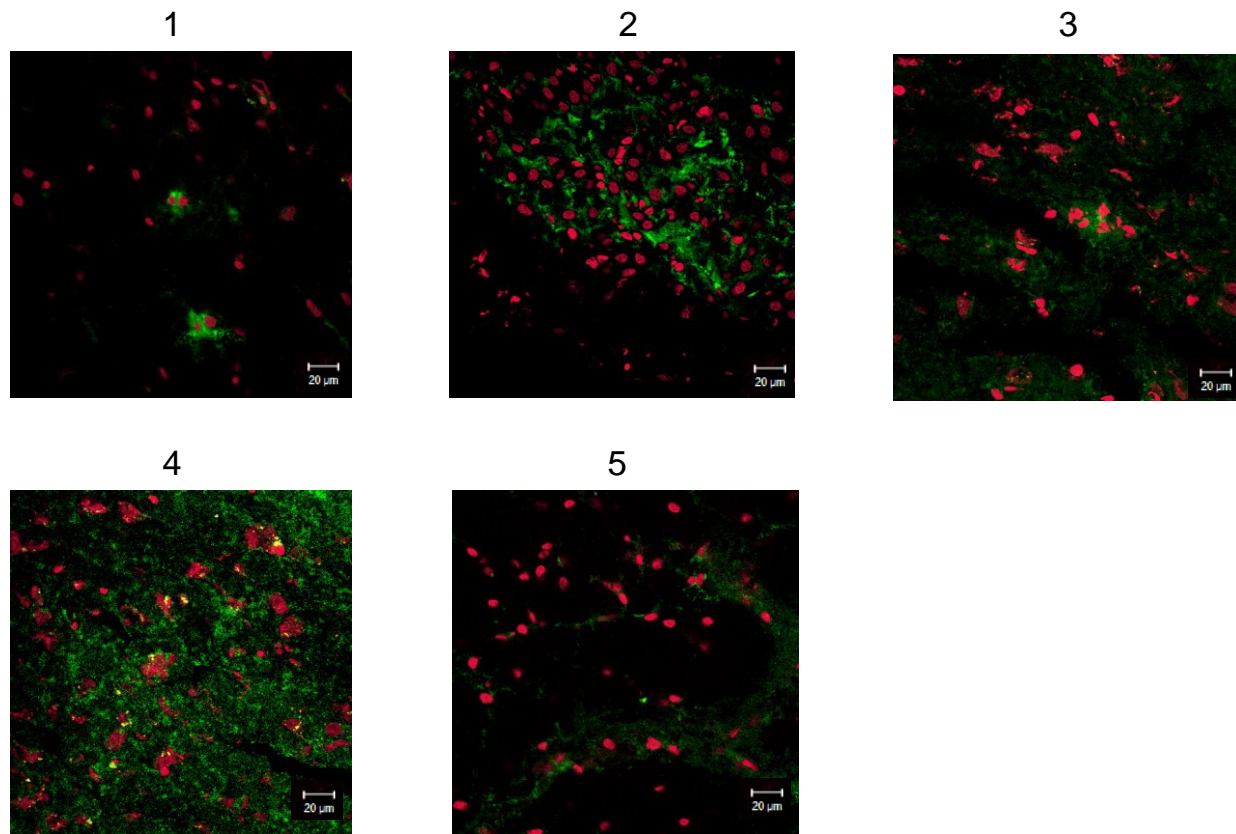


Figure 6.28 HSPA protein levels assessed using immunofluorescence in non-heat shocked rat brain tissue, PMI 0 h. (1) 0 h time course (2) 3 h time course (3) 6 h time course (4) 12 h time course (5) 24 h time course showing combined staining of nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green) Objective = x 40 magnification. Scale bar = 20μm.

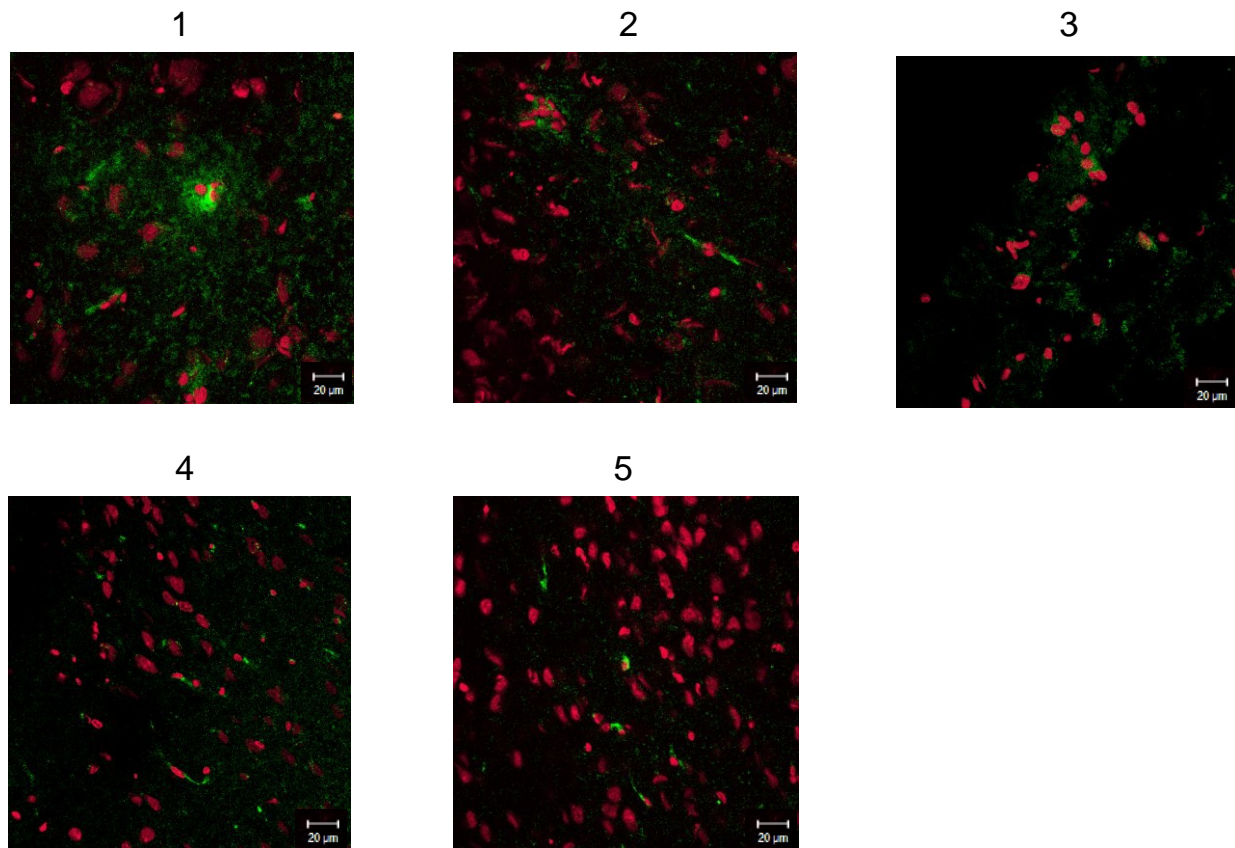


Figure 6.29 HSPA protein levels assessed using immunofluorescence in non-heat shocked rat brain tissue, PMI 3 h. (1) 0 h time course (2) 3 h time course (3) 6 h time course (4) 12 h time course (5) 24 h time course showing combined staining of nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green). Objective = x 40 magnification. Scale bar = 20µm.

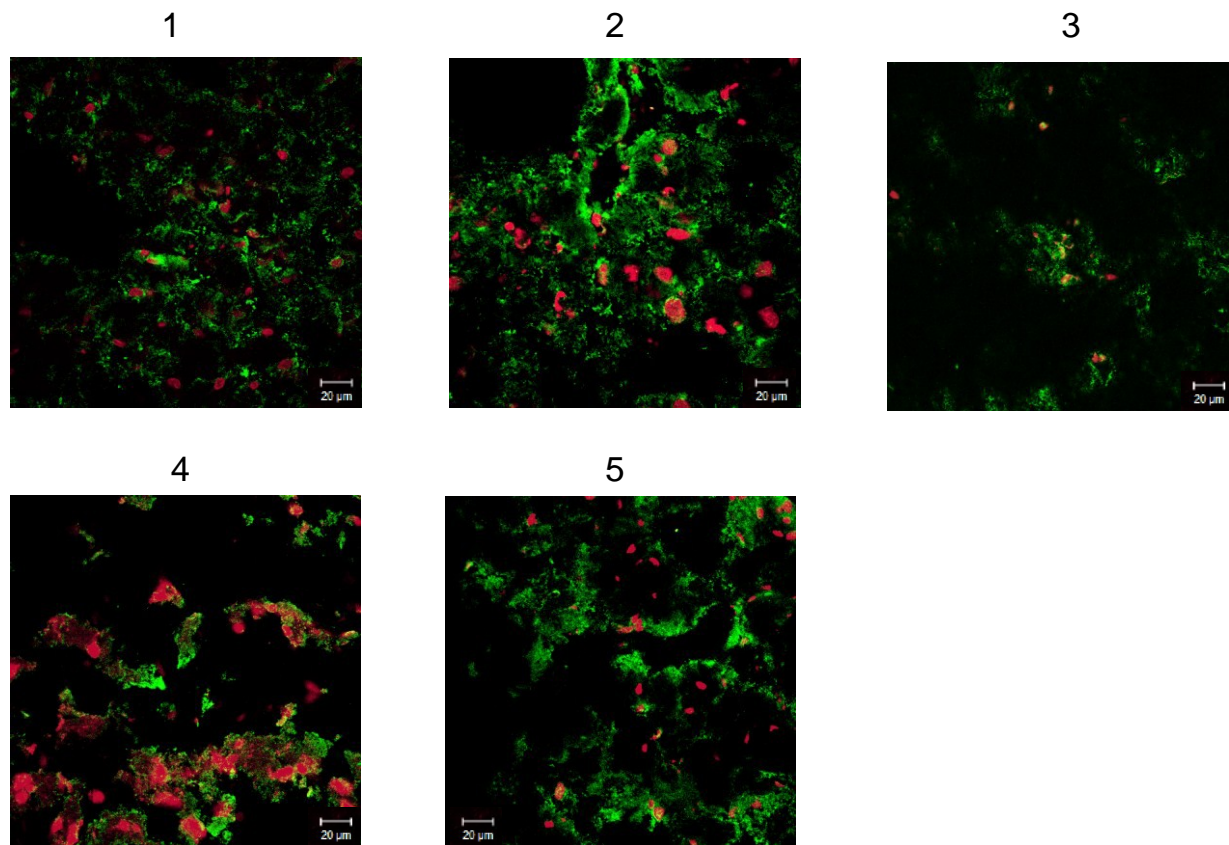


Figure 6.30 HSPA protein levels assessed using immunofluorescence in non-heat shocked rat brain tissue, PMI 6 h. (1) 0 h time course (2) 3 h time course (3) 6 h time course (4) 12 h time course (5) 24 h time course showing combined staining of nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green). Objective = x 40 magnification. Scale bar = 20µm.

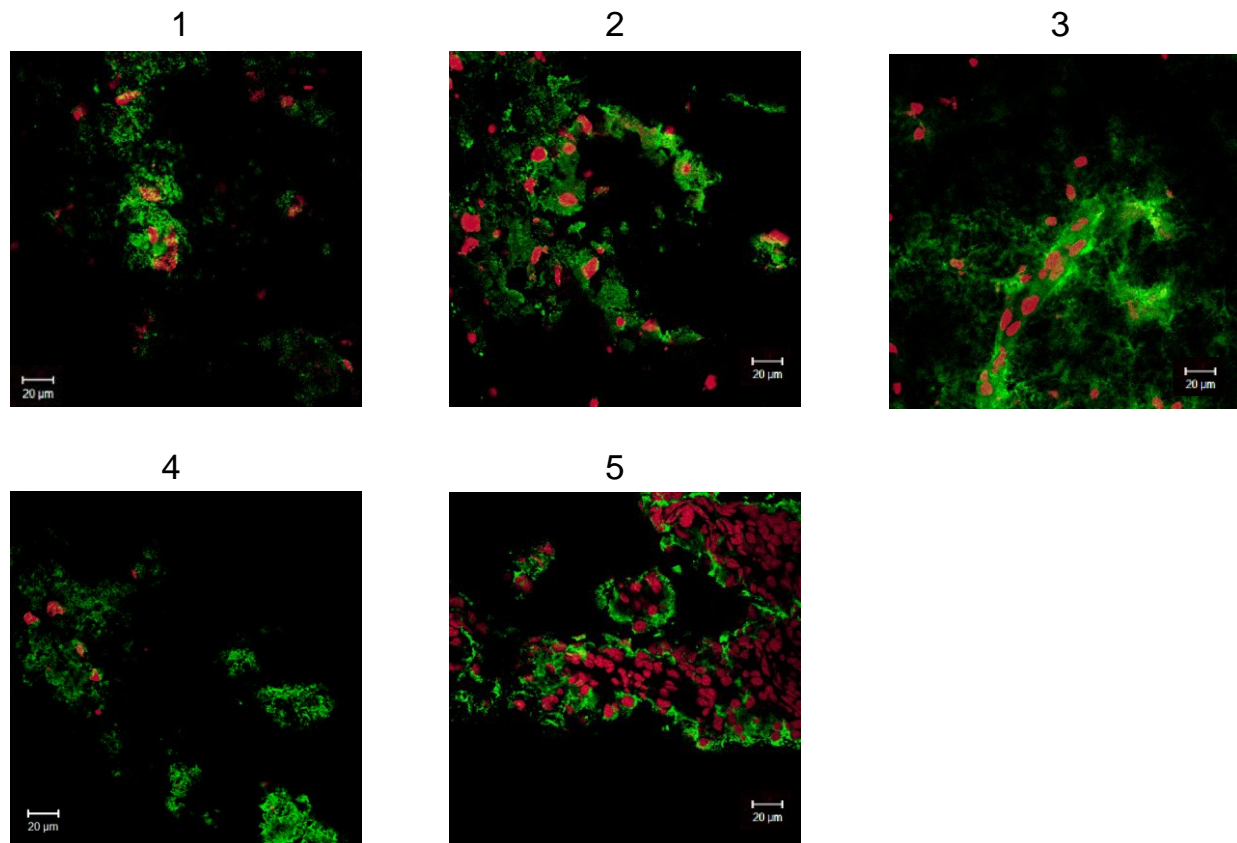


Figure 6.31 HSPA protein levels assessed using immunofluorescence in non-heat shocked rat brain tissue, PMI 12 h. (1) 0 h time course (2) 3 h time course (3) 6 h time course (4) 12 h time course (5) 24 h time course showing combined staining of nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

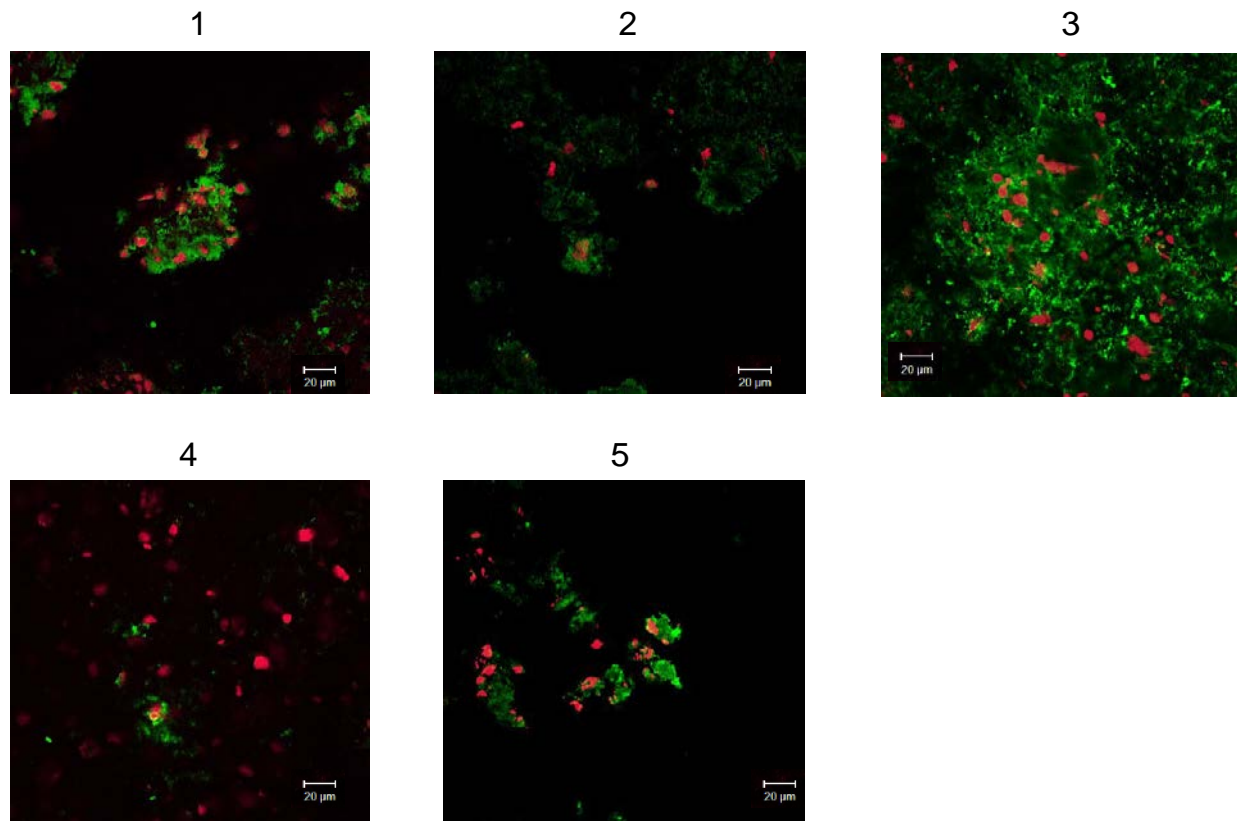


Figure 6.32 HSPA protein levels assessed using immunofluorescence in non-heat shocked rat brain tissue, PMI 24 h. (1) 0 h time course (2) 3 h time course (3) 6 h time course (4) 12 h time course (5) 24 h time course showing combined staining of nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

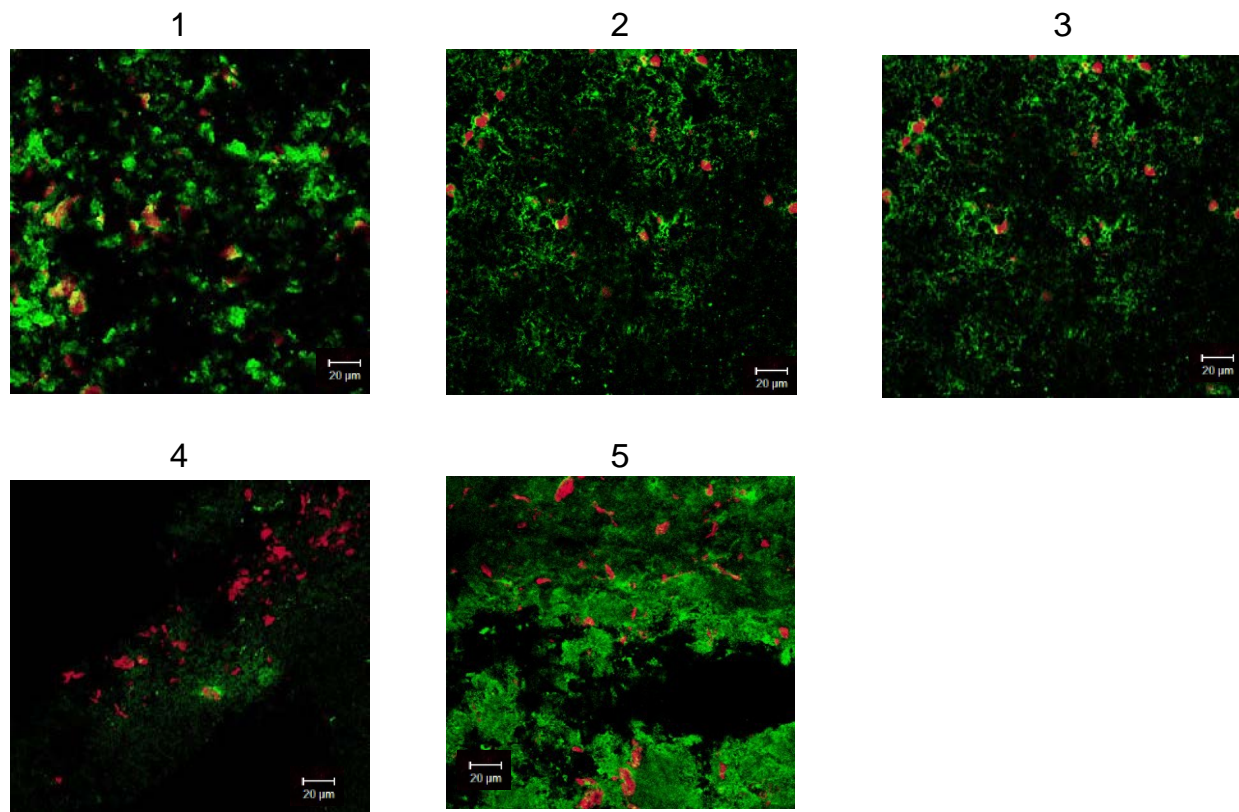


Figure 6.33 HSPA protein levels assessed using immunofluorescence in heat shocked rat brain tissue, PMI 0 h. (1) 0 h time course (2) 3 h time course (3) 6 h time course (4) 12 h time course (5) 24 h time course showing combined staining of nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

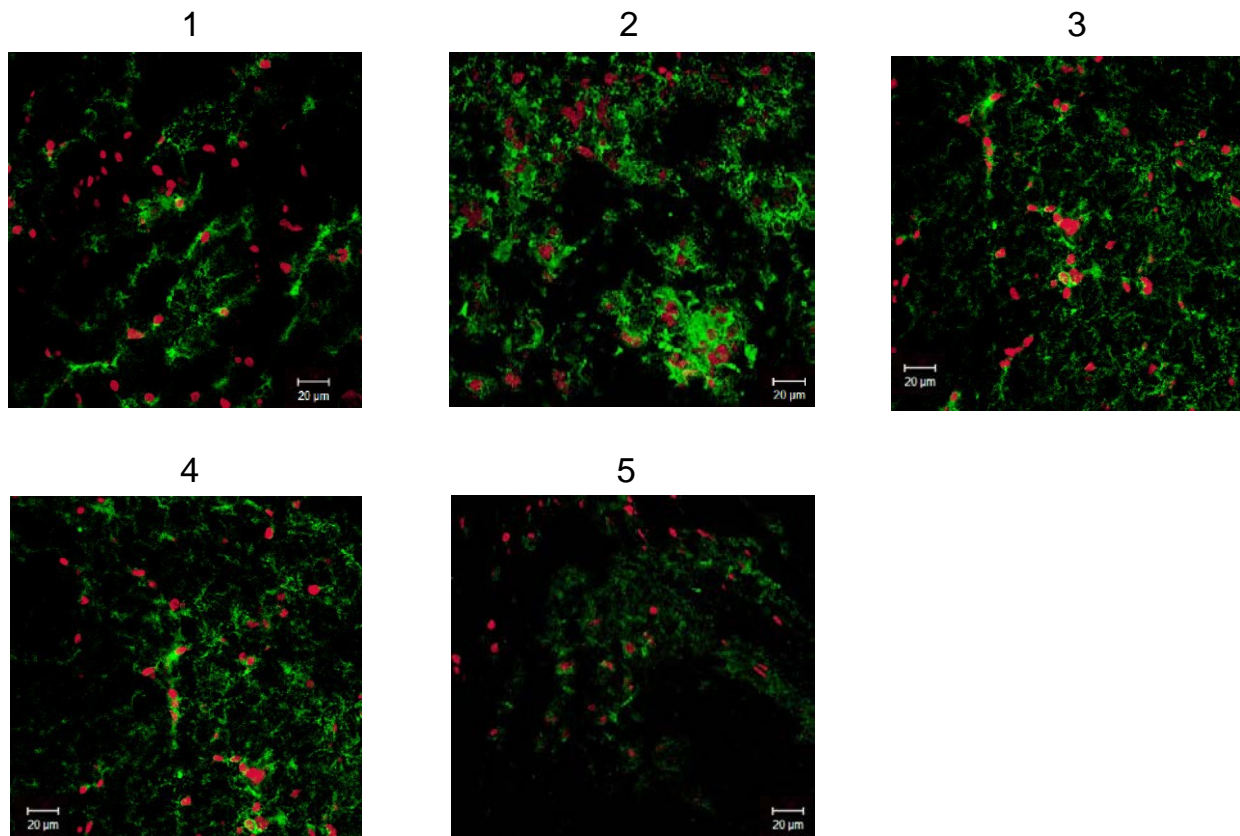


Figure 6.34 HSPA protein levels assessed using immunofluorescence in heat shocked rat brain tissue, PMI 3 h. (1) 0 h time course (2) 3 h time course (3) 6 h time course (4) 12 h time course (5) 24 h time course showing combined staining of nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

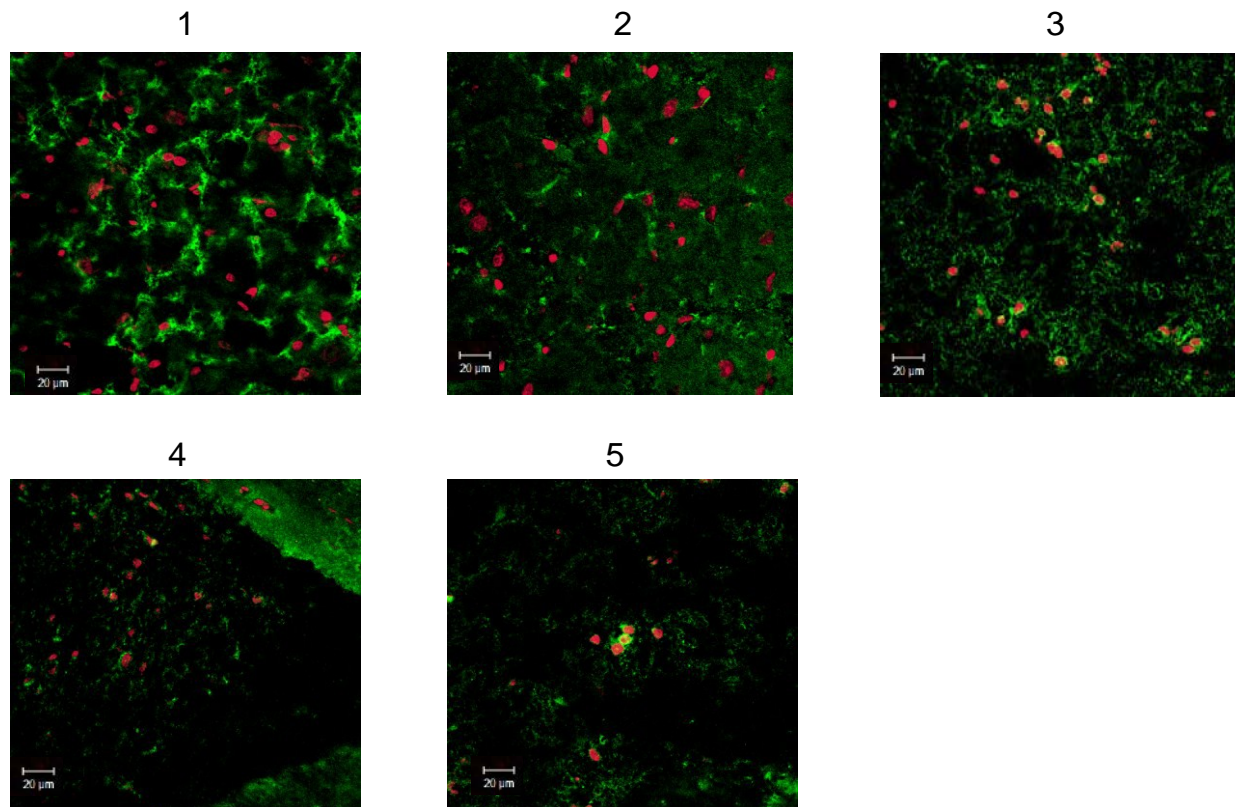


Figure 6.35 HSPA protein levels assessed using immunofluorescence in heat shocked rat brain tissue, PMI 6 h. (1) 0 h time course (2) 3 h time course (3) 6 h time course (4) 12 h time course (5) 24 h time course showing combined staining of nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

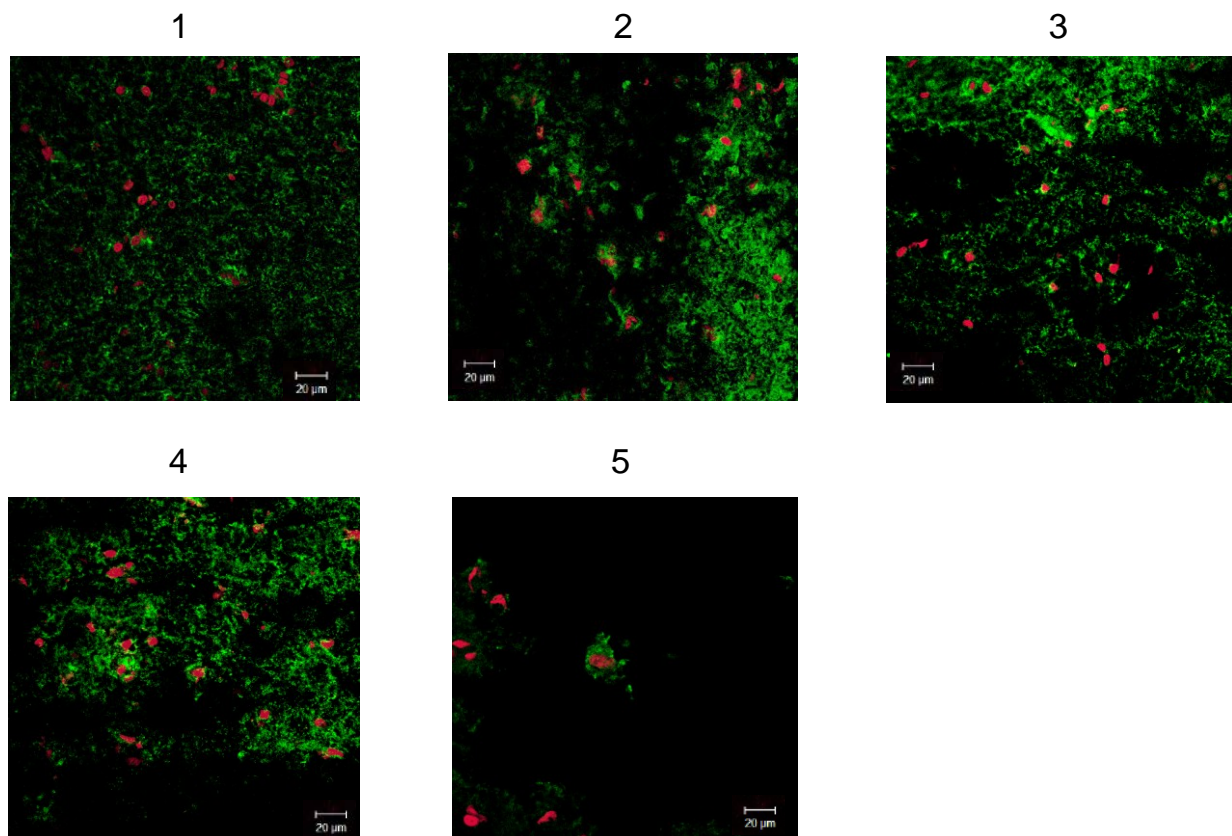


Figure 6.36 HSPA protein levels assessed using immunofluorescence in heat shocked rat brain tissue, PMI 12 h. (1) 0 h time course (2) 3 h time course (3) 6 h time course (4) 12 h time course (5) 24 h time course showing combined staining of nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green). Objective = x 40 magnification. Scale bar = 20µm.

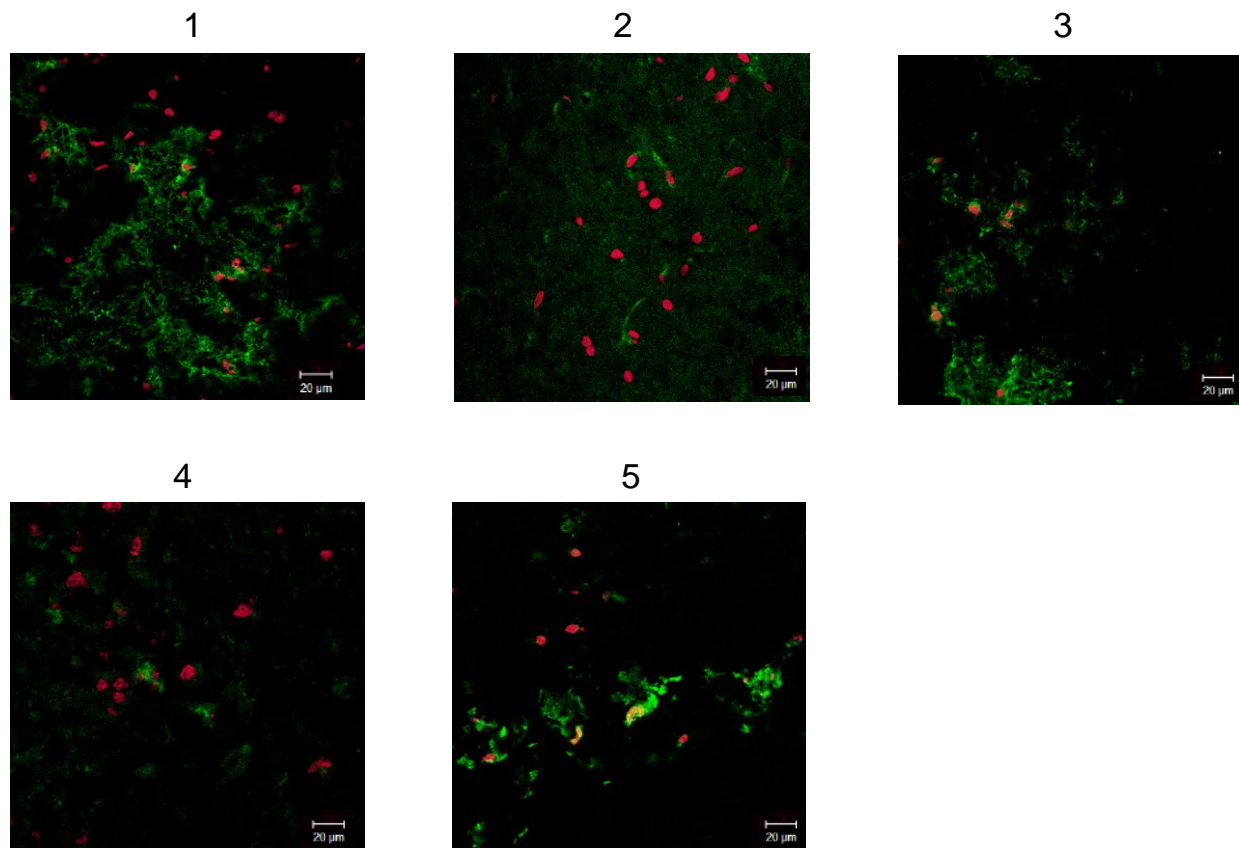


Figure 6.37 HSPA protein levels assessed using immunofluorescence in heat shocked rat brain tissue, PMI 24 h. (1) 0 h time course (2) 3 h time course (3) 6 h time course (4) 12 h time course (5) 24 h time course showing combined staining of nuclei labelled with propidium iodide (red) and primary antibody HSPA detected with Anti-mouse IgG FITC conjugated secondary antibody (green). Objective = x 40 magnification. Scale bar = 20μm.

Results from this investigation show that *HSPA* is expressed at low levels in normal brain tissue, but is highly expressed in brain tissue subjected to mild heat shock. In the non-heat shocked brain tissue, low level expression of *HSPA* was observed in the 0 h post-mortem interval at 0 h time course, followed by a gradual increase up to the 6 h time course interval, returning back to near basal levels at the 24 h time course interval. *HSPA* expression gradually decreased at 3 and 6 h post-mortem intervals however, *HSPA* transcripts substantially increased at the 12 h post-mortem interval. At the 24 h post-mortem interval *HSPA* transcripts were only present at low levels, below the initial basal levels observed at 0 h post-mortem 0 h time course interval. A higher level of *HSPA* expression was observed in samples taken at 0 h post-mortem that had been subjected to heat shock, compared to non-heat shock at each time course interval. *HSPA* transcripts were observed in the 0 h post-mortem interval at 0 h time course, followed by a gradual increase up to the 6 h time course interval, returning back to near basal levels at the 24 h time course interval. At 3 post-mortem, *HSPA* transcripts expressed high at 0 h time course with a gradual decline up to the 24 h time course. *HSPA* expression gradually decreased at 6 h post-mortem, however, as with non-heat shock, *HSPA* transcripts substantially increased at 12 h post-mortem. Again as with non-heat shock, *HSPA* transcripts were only present at low levels at the 24 h post-mortem interval, below or near to the initial basal levels observed at 0 h post-mortem 0 h time course interval.

The levels of *HSPA* transcripts in brain tissue that had been subjected to a mild heat shock, the first at 0 h time course followed by subsequent heat shocks at 3, 6 and 24 h time course intervals, showed a marked increase in *HSPA* expression in heat shocked brain tissue compared to non-heat shock within each post-mortem interval.

As expected, given that post-mortem and mild heat shock is a form of cellular stress, HSPA protein was detected in both the cytoplasm and the nucleus in all tissue samples at each post-mortem interval for both non-heat shock and heat shocked samples.

Chapter 7

Discussion

7.1. Discussion

7.1.1 Chapter 3 results

The target genes chosen for this study, *Homo sapiens* and *Rattus norvegicus* *HSPA*, both members of the heat shock protein family, are induced in response to various physiological and environmental stress conditions, or in response to certain diseases, such as cancer (Garrido *et al.*, 2006; Jolly and Morimoto, 2000; Lindquist, 1986; Morimoto, 1993; Powers *et al.*, 2009).

As discussed in chapter 3, section 3.3, the *HSPA* gene family contains three well characterized members: *HSPA1A*, *HSPA1B* and *HSPA1L*, which are located within the major histocompatibility complex class III region (MHC-III). In the human genome, they are located on the short arm of chromosome 6 (6p21.3), while in the rat genome, they are located on the short arm of chromosome 20 (20p12) (Milner and Campbell, 1990; Sargent *et al.*, 1989a; Sargent *et al.*, 1989). Although they contain similar nucleotide sequences, these three genes differ in their regulation. *HSPA1L* has no associated regulatory heat shock consensus sequence and is constitutively expressed at low levels, and is also induced by heat shock (Milner and Campbell, 1990). Therefore, this gene was excluded for the purposes of this study. Both *HSPA1A* and *HSPA1B* have been shown to encode identical protein products with 100% homology, and the genes also show 90% homology between their nucleotide sequences. Both have been shown to be expressed at high levels after heat shock, but only *HSPA1A* has been shown to be constitutively expressed at low levels (Milner and Campbell, 1990). After running a BLAST analysis for *HSPA1A* and *HSPA1B* and for the designed primers, results

indicated that both genes will be amplified at the same position and of the same product length for the designed primers (Appendix 9.26 and 9.27).

Studies have shown that, across a wide range of both human and rat tissues, the molecular integrity of 28S and 18S ribosomal RNAs and of some specific gene transcripts, such as interleukin-1 β (IL-1 β), can persist under post-mortem conditions for up to periods of 148 h in brain tissue (Inoue *et al*, 2002), making brain a suitable tissue for the purposes of this study. The molecular integrity of RNA is thus shown to be unlikely to change significantly in the early post-mortem period, up to 148 h in human and rat brain tissue, allowing the opportunity to elicit *de novo* gene expression of the *HSPA* gene in response to mild heat shock. Due to difficulties in obtaining post-mortem human brain tissue, rat brain tissue was chosen as an alternative source of tissue, and this was readily accessible from the Physiology Laboratory of this University. Sequence homology comparison between human and rat genes for *HSPA* indicated 73% homology between these two species for *HSPA* gene sequences and 96% homology for *HSPA* amino acid sequences, which also indicates that the rat is a comparable species to humans for the purposes of this study.

Primers for both *Homo sapiens* and *Rattus norvegicus* *HSPA* genes were designed using Primer 3 software. Melting curve analysis produced during RT-PCR showed only one defined peak indicating specific binding of primers to *HSPA*. This was confirmed by visualizing the resultant amplicons by agarose gel electrophoresis. Visualization of the amplicons showed the presence of a single band of the required product size. No primer-dimers or degradation were observed.

The choice of an appropriate internal standard is critical for quantitative protein and RNA analyses. Housekeeping genes are normally expressed in all cells and their expression levels should remain relatively constant under different experimental conditions. There is no single housekeeping gene that possesses stable expression levels under all experimental conditions. Therefore, it was necessary to characterize the suitability of any housekeeping gene chosen as an internal RNA control under particular experimental conditions where transcription effects are being tested. The chosen internal standard for this study was Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), a housekeeping gene which encodes a glycolytic enzyme that possesses diverse functions that are independent of its role in glycolysis (Said *et al*, 2009, 2007; Sirover, 1999). *GAPDH* is a multifunctional enzyme reportedly over-expressed in many tumours and malignant cells (Said *et al*, 2009, 2007).

Literature has shown that *GAPDH* expression is regulated by a variety of different factors such as insulin (Nasrin *et al*, 1990); calcium (Chao *et al*, 1990) and hypoxia (Graven *et al*, 1994). The transcription factor hypoxia-inducible factor-1 alpha (HIF-1 α), which regulates the expression of genes involved in glucose supply, growth, metabolism, redox reactions and blood supply, is undetectable under normoxic conditions because it undergoes rapid ubiquitination and proteosomal degradation (Rapisarda *et al*, 2002). Hypoxia, which is the inadequate delivery of oxygen to tissue causes an imbalance between *oxygen* demand and energy supply, results in the transcription of HIF-1 regulated hypoxia induced genes (Cangul, 2004; Said *et al*, 2007). A number of the proteins encoded by these genes are involved in adaptive responses to hypoxia, such as angiogenesis, erythropoiesis, cell proliferation and cell survival (Said *et al*, 2007; Schmid *et al*, 1998). It has been suggested that *GAPDH* expression is regulated as a consequence of the hypoxic development of the cellular

environment *in vitro* and that *GAPDH* mRNA expression was regulated during hypoxic events (Graven *et al*, 1994; Graven and Farber, 1998; Said *et al*, 2005; Zhong and Simons, 1999).

There are two types of hypoxia: transient and chronic hypoxia. Transient hypoxia is a temporary reduction in oxygen availability. The inadequate vascular geometry relative to the volume of oxygen-consuming tumour cells creates diffusion-limited oxygen delivery, resulting in chronic hypoxia (Greco *et al*, 2003). The synthesis of HIF-1 is up-regulated in response to hypoxia. In general, tumours often contain hypoxic regions, since tumour vasculature is dysfunctional and unable to meet the metabolic needs of rapidly proliferating cancer cells (Hanahan and Folkman, 1996). The extent of hypoxic regions in a tumour tissue depends on the arrangement, blood flow rate and blood oxygen content of microvessels, and on the tissue's oxygen consumption rate (Said *et al*, 2007).

Studies undertaken by Said *et al*, (2007, 2009) compared the expression of *GAPDH* with 18S RNA in relation to HIF-1 α under different oxygen concentrations of severe hypoxia, normoxia and re-oxygenation in human glioblastoma, low-grade astrocytoma and normal brain tissue samples, together with human glioblastoma cell lines. These results did not indicate any correlation between hypoxia induced HIF-1 α protein over-expression and *GAPDH* regulation on mRNA and protein level *in vitro* in human glioblastoma cell lines. *GAPDH* was not significantly regulated under hypoxic conditions in a panel of human tumour cell lines *in vitro*, and the expression of the HIF-1 gene was not altered after substitution of the *GAPDH* by the 18S RNA band (Said *et al*, 2005). Extended studies by Said *et al*, (2009) indicated significant mRNA over-expression of known hypoxia-regulated genes in glioblastoma, compared to low-grade

astrocytoma (Said *et al*, 2007). It can be suggested that from these studies that the regulation of *GAPDH* mRNA synthesis as a response to the hypoxic development in the tumour cell environment *in vitro* and *in vivo* occurs as a cell-specific post-transcriptionally regulated event and that there is no hypoxia-dependent regulation of *GAPDH* in astrocytic tumours *in vivo*. It can therefore be concluded that *GAPDH* is a suitable reference gene for studies in normal and tumour brain tissue and cell lines under hypoxic conditions.

Results described in chapter 4 for *GAPDH* showed that mRNA copy numbers were relatively consistent in NHA (approximately 146,500 copies per sample) 1321N1, (approximately 144,500 copies per sample), GOS-3, (approximately 147,500 copies per sample) and U87-MG, (approximately 147,500 copies per sample), confirming the routine comparability of *HSPA* results (Table 4.1). For *GAPDH*, mRNA copy numbers were relatively consistent in all brain tissue samples, at approximately 143,700 copies per sample), again confirming the relative comparability of *HSPA* results (Figure 4.3 and Table 4.6).

Results described in chapter 5 for *GAPDH* showed that mRNA copy numbers were relatively consistent in NHA-, (approximately 148,300 copies per sample), 1321N1, (approximately 147,400 copies per sample), GOS-3, (approximately 147,400 copies per sample) and U87-MG, (approximately 147,300 copies per sample), again confirming the relative comparability of *HSPA* results (Figure 5.2 and Table 5.1).

One of the most important technical considerations in this study was the quality of the isolated mRNA. Cellular mRNA is less stable than DNA due to the ubiquitous nature and stability of RNases compared with DNases, and so RNA is more susceptible to spontaneous degradation. In addition to the intrinsic RNases already present in cells and

tissues, samples may become contaminated by RNases from other sources, such as bench surfaces and laboratory equipment, and therefore rigorous laboratory procedures for aseptic technique were required. During this study, contaminating RNase activity was precluded by careful preparation of reagents, aseptic handling of samples and use of equipment. During this study only RNase free pipette tips, microfuge tubes and RNase free water was used and all equipment was pre-treated with RNase-Zap, a commercial surface decontamination solution that destroys RNases on contact (Sigma, UK), prior to use.

mRNA isolated from NHA, 1321N1, GOS-3 and U87-MG cell lines and rat brain tissue samples showed little or no degradation or contamination. The overall A_{260} / A_{280} value for isolated mRNA ranged between 1.8 – 2.0 in the cell lines and 1.6 – 1.8 in the brain tissue samples, which was deemed acceptable for the purpose of this study.

The primary and secondary antibodies used for flow cytometry and immunofluorescence were obtained from Abcam, UK. The primary antibody Monoclonal Anti-Heat Shock Protein 70 (HSP70) reacts specifically with HSP70 and recognizes brain HSP70 in both human and rat. Immunofluorescent staining using this antibody has demonstrated a rapid and reversible accumulation of the HSP70 protein within the nucleus of heat-stressed (42°C, 1 hr.) human fibroblasts (Morimoto, 1998).

The secondary antibody Goat polyclonal Secondary Antibody to Mouse IgG - H&L FITC, was recommended by Abcam. No dilution factor was recommended for either antibody, and following a number of pilot trials with different dilutions, 1:200 (1 µl of antibody in 200 µl of blocking solution) was the most efficient and gave the best results.

This study had six main objectives:

1. To characterize the level of *HSPA* expression in normal human cell lines and normal human brain tissue compared to glioma cell lines and tumourous brain tissue.
2. To characterize the level of *HSPA* transcription in response to hypoxia in normal and glioma cell lines.
3. To characterize the level of *HSPA* transcription in post-mortem brain tissue.
4. To characterize the level of *HSPA* transcription in response to heat shock applied to the brain tissue in the early post-mortem period.
5. To characterize the level of *HSPA* transcription in response to multiple heat shocks applied to the brain tissue in the early post-mortem period.
6. Validate if *HSPA* can be used as an early post-mortem marker.

7.1.2 Chapter 4 and 5 results

HSPA is expressed at low levels in normal cells and tissues, but its expression is highly induced by cancer. The results from this study show that *HSPA* is expressed at high levels in pre and post hypoxia treated glioma cells. The average *HSPA* mRNA copy numbers in the three glioma cell lines were approximately 6 fold higher than the normal cell line and the average *HSPA* mRNA copy numbers in glioblastoma tissue were approximately 1.8 and 9 fold higher than in the low grade glioma and normal tissues, respectively. In cancer, *HSPA* expression is induced to assist the stabilization of native proteins (Rohde *et al*, 2005). On the other hand, *HSPA* mRNA levels in the untreated NHA cells were relatively low in comparison to hypoxia treated cells. This is in agreement with previous reports such as Rohde *et al*, (2005), where significantly higher expression was reported in cancer tissue in comparison to normal tissue, supporting the

notion that the increased expression of *HSPA* is associated with cellular stress (Rohde *et al*, 2005).

Although prolonged exposure to extreme stress conditions can result in cell and tissue death, induction of heat shock protein synthesis can afford cytoprotection and stress tolerance against stress-induced molecular damage. Heat shock protein synthesis is induced by numerous stress stimuli resulting in the accumulation of non-native proteins.

The heat shock response in mammalian cells involves the activation of heat shock factor 1 (HSF1) in response to the presence of non-native proteins which require the action of molecular chaperones. HSF1 is present in both stressed and unstressed cells. In unstressed cells HSF1 is found in the cytoplasm as an inert monomer bound to HSPA and therefore does not show any transcriptional activity. In a stressed state when non-native proteins are detected, HSF1 translocates to the nucleus and oligomerises into a trimer that becomes inducibly phosphorylated and binds to the heat shock element upstream of the *HSPA* gene, resulting in stress-induced transcription (Santoro, 2000).

Given that cancer is a form of cellular stress, it is to be expected that *HSPA* protein should be observed in both the cytoplasm and the nucleus of glioma and normal cell lines under pre and post hypoxic conditions. Although *HSPA* protein was detected mainly in the cytoplasm in the normal cell line, after hypoxia treatment, migration of *HSPA* to the nucleus was observed. Increased synthesis of heat shock proteins to a level proportional to that of non-native proteins results in *HSPA* relocating to the nucleus and binding to the HSF1 transcriptional transactivation domain, thereby blocking transcription of heat shock genes (Santoro, 2000).

As mentioned previously, *HSPA* is expressed at low levels in normal cells and tissues, but its expression is highly induced by cancer. Results from this study showed that *HSPA* is highly expressed at mRNA levels in pre and post hypoxia treated glioma cells. The average *HSPA* mRNA copy numbers in the three glioma cell lines were approximately 6 fold higher than the normal cell line in both pre- and post-hypoxia treatment. In glioma cancerous cells, *HSPA* is induced to assist the stabilization of native proteins in an effort to maintain its functionality (Rhode *et al*, 2005). On the other hand, *HSPA* mRNA levels in the untreated NHA cells were relatively low in comparison to hypoxic cells. A previous citation, Rohde *et al*, (2005), reported a significantly higher expression in breast cancer tissue in comparison to normal, supporting the notion that the increased expression of *HSPA* is associated with stress (Rhode *et al*, 2005)

High fluorescence intensity of *HSPA* observed in 1321N1, GOS-3 and U87-MG cells exposed to hypoxia showed a 3-fold increase compared to NHA cells, which suggests that hypoxia can activate HSF1 in many cell types, resulting in an increased expression of *HSPA*. Production of heat shock proteins in response to hypoxia depends on the cell type. In some endothelial cells, such as human microvascular (HMEC1), *HSPA* expression in response to hypoxia is down-regulated (Oehler *et al*, 2000). In contrast, *HSPA* expression is up-regulated in human hepatoma (HEP G2) (Patel *et al*, 1995) and human proximal tubular epithelial cells (Turman *et al*, 1997).

In neoplasms, *HSPA* expression has been associated in the regulation of apoptosis as a modulator of p53 function in the immune response against tumours and multidrug resistance (Ciocca *et al*, 1993; Soussi and Lozano, 2005). Increased *HSPA* expression enhances the resistance of cells to apoptosis which elevates immunity due to their

highly immunogenic properties. Thus, during carcinogenesis, the expression of heat shock proteins will be altered in many tumour types (Schmitt *et al*, 2007). High expression levels of HSPA protein were detected in the cytoplasm of bladder carcinomas, but no correlation was found between the expression level, grade of tumour, disease phase and patient outcome (Lebret *et al*, 2003).

Glioblastoma patient survival rate is considerably short, as indicated in Table 4.5 section 4.2.3. The survival period of patients from diagnosis to death ranged between 8 to 12 months with only one patient exceeding this period. The sample size used in this study indicating survival period across the patient sample data range is too small to make any significant conclusion. Future studies need to be undertaken using a larger sample base to include a larger variance of survival rate together with a wider range of cancer grade. However, from this initial study, the notion that expression of *HSPA* appears to be grade related is consistent with the results obtained in Figure 4.5.

Results from this part of the study showed that *HSPA* was similarly expressed at high levels in all three glioma cells lines *in vitro*, (Figure 4.1) however in the human brain tissue samples *HSPA* was predominantly expressed in malignant astrocytomas *in vivo* especially in glioblastomas (Figure 4.2), the most aggressive and phenotypically transformed of the gliomas. Although the results indicated an apparent grade related expression in the human brain tissue samples, this was not replicated in the three glioma cells lines. A possible explanation for this is that *in vitro* (cell lines) experiments, in cellular biology, are conducted outside of the organisms which do not correspond to the same conditions inside the organism (*in vivo*) which may lead to results that do not correspond to the same situation that arises in a living organism. For example, *in vivo*, tumour cells can release a variety of degradative enzymes, and enhanced proteolytic

activity which has been associated with tumour growth, angiogenesis and invasiveness (Yamamoto *et al*, 1996).

Significant differences were shown in *HSPA* mRNA expression levels between normal and the three glioma cells (Table 4.2), however no differences were observed at the protein level (Table 4.9), indicating that the level of HSPA protein did not directly correlate with the level of *HSPA* mRNA, suggesting *HSPA* expression in the glioma cell lines might be regulated either at the transcriptional level by a mechanism governing message stability or at the posttranscriptional level.

Although heat shock protein levels are not informative as diagnostic markers, they are useful biomarkers for carcinogenesis in some tissues (Ciocca and Calderwood, 2005). The high levels of *HSPA* expression detected in the majority of glioblastoma tissues, compared to low grade gliomas and normal tissues, may be taken as an indicator of its potential use as a biomarker for carcinogenesis in glioma. Elevated levels of *HSPA* have been related to malignancy, metastasis, poor prognosis and resistance to therapeutic strategies, including chemotherapy or radiation in glioblastoma, breast, bladder, endometrial and cervical carcinomas (Piura *et al*, 2002; Syrigos *et al*, 2003; Thanner *et al*, 2003). In contrast, a high level of *HSPA* expression has been associated with good prognosis in melanoma, pancreatic and renal carcinomas (Ricanidis *et al*, 2001; Sagol *et al*, 2002; Santarosa *et al*, 1997), suggesting possible tissue specificity.

Previous studies have shown that silencing of *HSPA* isoforms produces variable effects on cell viability, depending on the cell line used. Furthermore, silencing of one isoform heat shock cognate 70 (*HSPA8/HSC70*) leads to concurrent induction of the other isoform heat shock protein 72 (*HSPA/HSP72*) in glioblastoma, colon and ovarian cancer

cell lines (Powers *et al*, 2008). By silencing the *HSPA* isoforms, heat shock protein 90 (*HSPC1^a/HSP90*) function was modulated and also induced apoptosis in cancer cells. *HSPC1^a/HSP90* inhibition has been a promising target for cancer inhibition over the last decade. Thus, it can be postulated that a multi-target approach should be adopted modulating *HSPA* and *HSPC1^a/HSP90* function, for the future (Powers *et al*, 2009).

BAG1 and HSPA protein synthesis is up-regulated during the early post-mortem period (up to 4 hours) (Curcio *et al*, 2006; Torres *et al*, 2004). In cases of unexplained deaths one of the most important factors is accurate and precise determination of time since death, referred to as the post-mortem interval (PMI). Various methods are currently employed to determine PMI: temperature measurements post-mortem, biochemical markers and post-mortem muscle proteolysis (relaxation of muscles following rigor mortis). In many types of cancers, *HSPA* expression becomes dysregulated, resulting in elevated levels under stress conditions; furthermore, HSPA protects cancer cells from apoptosis (Calderwood, 2005). As HSPA1A protein is up-regulated in diseases such as cancer, Alzheimer's and Parkinson's disease (Petrucelli *et al*, 2004), in order to use HSPA as an early post-mortem marker, the possible disease of the tissue also needs to be considered as a potential parameter.

7.1.3 Chapter 6 results

This part of the study had three main phases. Firstly, RNA was extracted from post-mortem rat brain tissue following post-mortem intervals of 0, 3, 6, 12 and 24 h at subsequent time course intervals of 0, 3, 6, 12 and 24 h, to determine when expression of *HSPA* was induced to characterise the persistence of *HSPA* transcripts in cells and as an indicator of *de novo* expression in these post mortem samples. The second part of

the study characterised the level of transcription in response to post-mortem cellular changes, and in response to heat shock applied to the rat brain tissue at 0 h time course interval within each post-mortem interval. The third part of this study characterised the levels of transcription in response to post-mortem cellular changes, and as a result of heat shock applied to the rat brain tissue at 0 h, and then again at 3, 6 and 24 h time course intervals within each post-mortem interval.

In the initial part of the study, it was determined that *HSPA* gene transcripts were present at each post-mortem interval (0, 3, 6, 12 and 24 h), which supported findings from previous literature (Blake *et al*, 1989; Inoue *et al*, 2002; Pardue *et al*, 2007). Low level expression of *HSPA* was observed in the 0 h post-mortem interval at 0 h time course (copy number $19.21 \pm .923$), followed by a gradual increase up to the 6 h time course interval (copy number 54.94 ± 4.47) and then returning to near basal levels at the 24 h time course interval (copy number 17.40 ± 2.79). No *HSPA* expression was expected at the 0 h time course interval, as *HSPA* is only induced by stress conditions, but, the low levels of *HSPA* transcripts may have resulted from induced stress during the initial sacrifice procedure of the rat and during the subsequent excision of the brain. As expected, *HSPA* expression gradually decreased at 3 and 6 h post-mortem intervals. However, *HSPA* transcripts substantially increased at the 12 h post-mortem interval by approximately 2 fold at 0 h time course, 6 fold at 3 h time course, 3 fold at 6 h time course, 3 fold at 12 h time course and 2.5 fold at 24 h time course. At the 24 h post-mortem interval *HSPA* transcripts were only present at low levels, below the initial basal levels observed at 0 h post-mortem 0 h time course interval.

In the second stage of this study, the levels of *HSPA* transcripts in brain tissue that had been subjected to a mild heat shock at the 0 h time course interval within each post-

mortem interval were compared to *HSPA* transcript levels in the non-heat shocked brain tissue. There was an increased level of *HSPA* expression following heat shock, although maximum levels of expression were seen at different time points through the post mortem period used in experiments. Maximum expression was observed at: 6 h within 0 h PMI; at 0 h within 3 h PMI; 24 h within 6 h PMI; 12 h within 12 h PMI and 3 h within 24 PMI. Under non-heat shock conditions, *HSPA* transcripts were observed in the 0 h post-mortem interval at 0 h time course (copy number 21.34 ± 1.41), followed by a gradual increase up to the 6 h time course interval (copy number 83.47 ± 9.03) returning back to near basal levels at the 24 h time course interval (copy number 31.03 ± 3.90). At the 3 post-mortem, *HSPA* transcripts expressed high at 0 h time course (copy number $57.96 \pm .00$) with a gradual decline up to the 24 h time course (copy number 41.59 ± 7.51). *HSPA* expression gradually decreased at the 6 h post-mortem interval, but as with non-heat shock, *HSPA* transcripts substantially increased at the 12 h post-mortem interval by approximately: 1.25 fold at 0 h time course, 1.25 fold at 3 h time course, 2 fold at 6 h time course, 3 fold at 12 h time course and 0 fold at 24 h time course. Again, as with non-heat shock, *HSPA* transcripts were only present at low levels at the 24 h post-mortem interval, below or near to the initial basal levels observed at 0 h post-mortem 0 h time course interval.

In the third stage of this study, the levels of *HSPA* transcripts in brain tissue that had been subjected to a mild heat shock, the first at 0 h time course followed by subsequent heat shocks at 3, 6 and 24 h time course intervals, were accessed to determine whether the application of subsequent heat shocks would increase *HSPA* expression in a thermotolerant state. In all cases there was a marked increase in *HSPA* expression in heat shocked brain tissue compared to non-heat shock. This phenomenon, known as thermotolerant preconditioning, is a cellular adaptation resulting from a single severe

non-lethal exposure to heat that allows cells to better survive subsequent potentially lethal heat stress episodes, and has been reported previously (Lindquist and Craig, 1988; Moseley, 1997; Pardue *et al*, 2007; Theodorakis and Morimoto, 1987; Theodorakis *et al*, 1999; Tolson and Roberts, 2005). The characteristics for thermotolerance are dependent on survival of the cell(s) exposed to an otherwise lethal heat stress, synthesis of heat shock proteins and a short duration of the thermotolerant state (Moseley, 1997). The response of cells to such stress conditions is dependent on the concentration of *HSPA* in the cell prior to stress and on the severity of the stress, based upon its intensity and duration (Lindquist and Craig, 1988; Mosser and Morimoto, 2004; Parsell and Lindquist, 1994).

HSPA is constitutively expressed under normal conditions and inducibly in response to cellular stress (Lindquist 1986). Under normal conditions, *HSPA* is constitutively expressed at relatively low but constant levels. Under conditions of stress, such as death or heat shock, weak hydrogen and van der Waals forces that hold cellular proteins in their conformational shape become weakened, resulting in protein denaturation, incorrect protein aggregation and mis-folding of newly synthesized proteins (Hartl and Hayer-Hartl, 2002; Nollen and Morimoto, 2002). Under such conditions, *HSPA* genes are induced in order to protect proteins and enhance cell survival (Calderwood *et al*, 2006; Mosser and Morimoto 2004; Powers *et al*, 2009). In addition to the above, *HSPA* plays a strong part in cytoprotection, allowing cells to adapt and to survive under hostile or otherwise lethal conditions (Calderwood *et al*, 2006; Mosser and Morimoto 2004; Parcellier *et al*, 2003; Powers *et al*, 2009).

The induction of *HSPA* production in response to stress conditions involves an increase in active heat shock factor 1 (HSF1) which is released from *HSPA* during an initial

stress, and subsequently binds to the heat shock element (HSE) in the *HSPA* gene promoter region (Fernandes *et al*, 1994; Morimoto *et al*, 1994; Wu *et al*, 1994,). Inducible transcription of the *HSPA* gene requires the *de novo* binding of the HSE by a heat shock transcription factor 1 (HSF1) protein, which is ubiquitously synthesised and plays a major role in the stress induced expression of the *HSPA* genes (de Thonel *et al*, 2011). *HSPA* synthesis is regulated at the transcriptional level by the heat shock factor 1 (HSF1) which assists, not only the long term induction of the *HSPA* gene, but also the regulation of gene expression and developmental processes (Morimoto, 1998; Sreedhar *et al*, 2004). This response protects the cells against further insults, and has been demonstrated in a number of *in vivo* and *in vitro* studies (Krishnan *et al*, 2006; Pespeni *et al*, 2005; Wagstaff *et al*, 1999). Although the production of heat shock proteins results in the protection of cells from the effects of further stresses, they are deemed toxic if present in the cells for any prolonged period (Theodorakis *et al*, 1999).

The brain tissue that was made thermotolerant by the application of multiple heat shocks contained lower levels of *HSPA* transcripts in the 0 and 3 post-mortem intervals compared to non-heat shock and to single heat shock, suggesting there is a regulation of *HSPA* synthesis that is dependent on the levels of *HSPA* existing within the cell (Kregel, 2002). Studies have demonstrated that inhibition of transcription during the conditioning heat stress allows the maintenance of thermotolerance (Bader *et al*, 1992). Induction of *HSPA* was rapid with high levels of the induced transcripts present at 6 h post-mortem intervals. Levels were substantially higher than those observed in non-heat shock and single heat shock samples, which supports work undertaken by Blake *et al*, (1990) and Theodorakis *et al*, (1999).

No significant differences were observed between the level of translation of *HSPA* mRNA in non-heat shock compared to the translation level in heat shocked tissue as shown in section 6.2.1. It can be concluded that heat shock is not necessary for the efficient translation of *HSPA* mRNA. Results from this study have shown that the transcription of the *HSPA* gene is rapidly and transiently activated in tissue subjected to a single heat shock. During heat shock, *HSPA* transcription levels rapidly increase and then decline after 6 h, section 6.2.1, Figure 6.1. The high levels of *HSPA* mRNA in the multiple heat shocked tissue is maintained through effects of message stability, due to the increase in heat shock factor (HSF) that is released from *HSPA* during the initial stress, which subsequently binds to the heat shock element (HSE) in the *HSPA* gene promoter region (Morimoto *et al*, 1994). Heat shock has an effect on the stability of *HSPA* mRNA. In non-heat shocked tissue, the half-life of *HSPA* mRNA, as stated by Theodorakis and Morimoto (1999), is approximately 50 min, but in heat shocked tissue the stability increases by at least 10-fold (Theodorakis and Morimoto, 1987). Previous studies have shown that *HSPA* mRNAs rapidly degrade during recovery from heat shock, which support the findings in this study (Blake *et al*, 1990; Pardue *et al*, 2007; Theodorakis *et al*, 1999; Theodorakis and Morimoto, 1987).

Tissue that was subjected to multiple heat shocks showed limited expression of *HSPA* compared to the single heat shock in the early post-mortem intervals 0 and 3 h. This could be due to a decrease in *HSPA* transcription and an increase in *HSPA* mRNA degradation, thus increasing cellular levels of *HSPA* in the recovery period, when returned to non-heat shock conditions 37°C. In contrast, during the heat shock period, no significant differences were observed in the levels of *HSPA* transcription in multiple heat shock compared to non-heat shock in the early post-mortem period 0 and 3 h. *HSPA* mRNA was observed to be less stable in multiple heat shocked tissue, compared

with the control non-heat shocked tissue. As stated by Theodorakis *et al*, (1999), a possible reason for this is that, under normal conditions, levels of *HSPA* mRNA are unstable, but during heat shock the mRNA becomes more stable due to mRNA degradation being affected by stress. In the tissue subjected to heat shock, mRNA degradation begins immediately after returning to non-heat shock conditions, and therefore it could be postulated that mRNA degradation is accelerated in tissue subjected to heat shock which would result in a reduced *HSPA* mRNA half-life.

Results have also shown that the levels of HSPALA proteins were higher in non-stressed and stressed (tumourous) human brain than those in non-stressed and stressed (heat shock) rat brain which supports the findings from Pardue *et al*, (2007). Consistent with the presence of the *HSPA* protein, *HSPA* mRNA levels were also found to be higher in non-stressed human brain than in non-stressed rat brain. This supports the overall conclusion by Pardue *et al*, (2007) that *HSPA*/HSP70 protein levels in brain tissue are significantly higher in humans who die suddenly and are not subjected to agonal stresses pre-mortem, or from disease such as brain tumours, compared to rats that are either non-stressed pre-mortem or that have been subjected to heat shock post-mortem.

Given that death and heat shock are both forms of cellular stress, it is to be expected that *HSPA* protein was observed in both the cytoplasm and the nucleus of samples subjected to these conditions. *HSPA* protein was detected mainly in the cytoplasm in non-heat-shocked brain tissue in the early post-mortem period with gradual migration into the nucleus over time. In the heat shocked post-mortem brain tissue, *HSPA* protein was observed in both the cytoplasm and the nucleus. Increased synthesis of heat shock proteins in response to heat shock, results in *HSPA* relocating to the nucleus and

binding to the HSF1 transcriptional transactivation domain, thereby blocking transcription of heat shock genes (Santoro, 2000).

7.2 Summary

Overall, we may draw a number of conclusions from this study. Firstly, *GAPDH* showed relatively consistent expression in human normal and tumourous cell lines and tissue samples under normal and hypoxic conditions, and also in rat brain tissues at different post-mortem intervals under normal and heat shock conditions. For *Homo sapiens GAPDH*, the average transcript copy number for normal and tumourous cell lines was approximately 146,500 copies per 100 ng of extracted mRNA, and for normal and tumourous brain tissues samples this was similar at approximately 144,000 copies per sample. For *Rattus norvegicus GAPDH*, levels were higher than for human, with an average of 268,300 copies per 100 ng of extracted mRNA. This study supports previous literature (Barber *et al*, 2005; Said *et al*, 2005, 2007, 2009) in showing that the expression of *GAPDH* is not up or down regulated by post-mortem, cancer, hypoxic or heat shocked conditions, and therefore *GAPDH* is confirmed as a suitable candidate control gene for the purposes of this study.

Secondly, it was found that it was possible to isolate mRNA fragments from post mortem tissue up to 24 hours post mortem, which is in agreement with the previous report of Inoue *et al*. (2002).

Thirdly, *HSPA* is expressed at low levels in normal cell lines, at approximately 1,420 copies, and normal brain tissue samples, at approximately 200 copies, but its expression is highly induced by cancer in glioma cell lines, with approximately 5,360 copies and in glioma brain tissue samples, with approximately 1,600 copies, as shown in Figure 7.1.

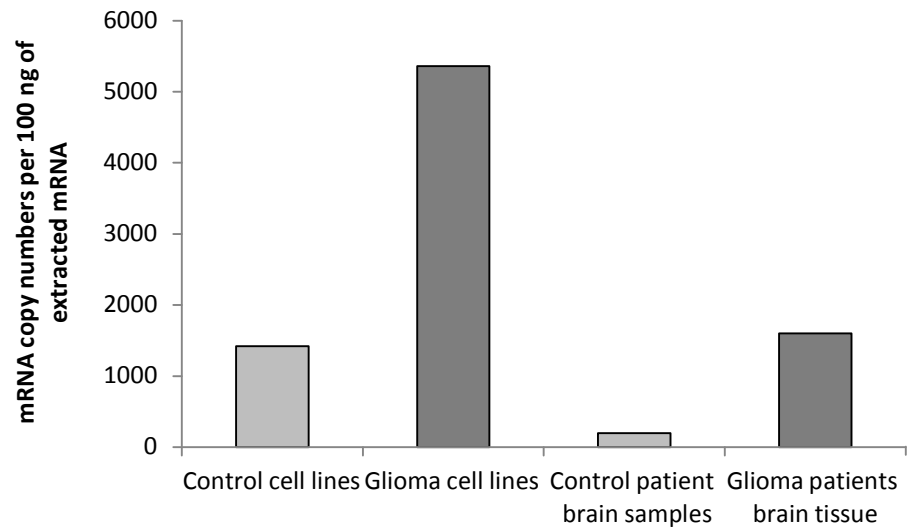


Figure 7.1 Histogram showing the average *HSPA* transcript levels from experiments using in control, glioma cell lines and v glioma brain tissue samples. *HSPA* can be seen to be more highly expressed in glioma cell lines and glioma brain tissue samples than in normal cell lines and normal brain tissue samples.

The results from this study also show that *HSPA* is expressed at low levels in pre and post hypoxia treated normal cells, (approximately 1,420 and 1,100 copies per 100 ng of extracted mRNA respectively) but is highly expressed in pre and post hypoxia treated glioma cells, (approximately 8,200 and 7,900 copies per 100 ng of extracted mRNA respectively), as shown in Figure 7.2.

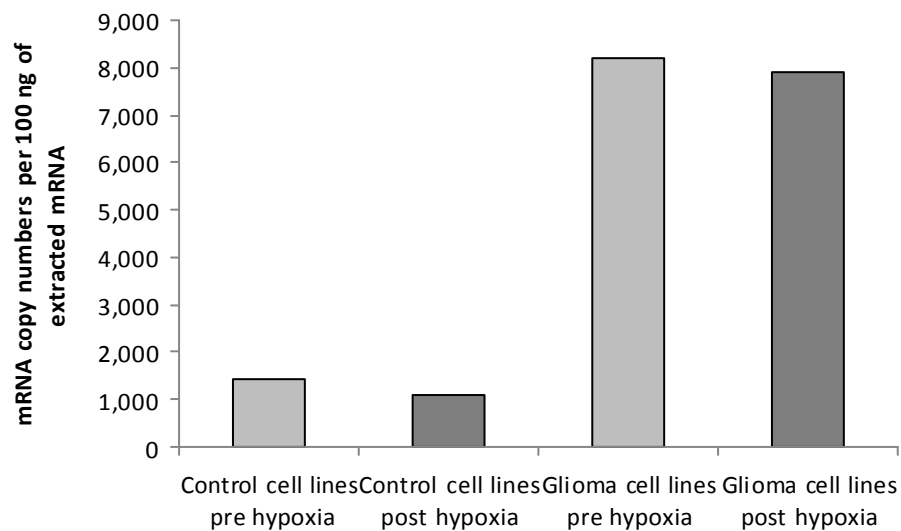


Figure 7.2 Histogram showing the average *HSPA* transcript levels in control and glioma cell lines under pre and post hypoxia conditions. *HSPA* can be seen to be more highly expressed in glioma cell lines under pre and hypoxia conditions compared to low *HSPA* expression in the normal cell lines under pre and post hypoxia conditions.

Hypoxia is a condition resulting from decreased cellular oxygen levels in tissues. In cellular hypoxia, tumours require additional blood vessels, a condition which is evident in many neoplasms. Hypoxia induces transcriptional activation of many genes that affect cellular metabolism and also promotes neoangiogenesis (the formation of new or recent blood vessels) (Duffy *et al*, 2003). Cells are able to sense and respond to

decreased oxygen levels through the conserved hypoxic response pathway involved in tumourigenesis. Exposure to decreased oxygen levels initiates the hypoxic response pathway by the regulated expression of hypoxia inducible transcription factor-1 (HIF-1). Many of the hypoxia-inducible genes are up-regulated during hypoxia, thus increasing oxygen transport to hypoxic tissues by promoting angiogenesis and also promoting cell proliferation and survival. However, failure to adapt to decreased oxygen levels will ultimately result in cell death via apoptosis (Bruick, 2003). Angiogenesis is associated with metastasis, and as tumours grow, some cells become detached from the tissues nutrient supply limiting the delivery of oxygen and nutrients to those cells (Duffy *et al*, 2003). This results in cellular hypoxia and metabolic stress inducing changes in transcriptional regulation, promoting growth of highly permeable blood vessels and facilitating the passage of tumour cells into the circulatory system. The response of cancer to hypoxia not only maintains the survival and growth of tumours, but also promotes tissue invasion and metastasis through angiogenesis (Duffy *et al*, 2003).

As predicted, HSPA protein was detected in both the cytoplasm and the nucleus of the three glioma cell lines under pre and post hypoxic conditions. Although HSPA protein was detected mainly in the cytoplasm in the normal (NHA) cell line pre-hypoxia, after hypoxia treatment, migration of HSPA protein to the nucleus was observed.

Although this research sheds light on the localisation and production of heat shock proteins in cells and supports a correlation between cancer and hypoxia environments, further research into glioma cells under hypoxic conditions is required to underpin the regulating mechanisms of HSPA in different cell types under such conditions. If cancer cells are expressing *HSPA* at high levels, then hypoxic conditions will have little or no further effect on these cells. Initially, tumours grow under non-hypoxic conditions, but

under hypoxic conditions, enhanced necrosis at the core of the tumour mass may be observed. Given that not all glioblastomas show the same degree of angiogenesis and necrosis, further research to address the localisation of *HSPA* in relation to a hematoxylin and eosin histology diagnosis (H and E) referring to angiogenesis and necrosis needs to be carried out. Furthermore, comparisons should be made between *HSPA* transcripts and protein levels after hypoxia with HIF to address whether cancer cells respond to hypoxia at HIF target gene levels. The glioma tissues indicate a correlation between *HSPA* expression and the grade of cancer, suggesting its possible application as a biomarker. This may also advocate the use of *HSPA* as a possible molecular target for therapy which may prolong glioma patient's survival in the future. However, different brain regions may show differences in *HSPA* levels, and thus additional work should be carried out to compare *HSPA* expression levels from various regions within the brain. The present study suggests that *HSPA* is up-regulated in glioma and it may be a suitable prognostic biomarker. .

HSPA was expressed at low levels in normal brain tissue, but was highly expressed in brain tissue subjected to mild heat shock. In the non-heat shocked brain tissue, low level expression of *HSPA* was observed in the 0 h post-mortem interval at 0 h time course, followed by a gradual increase up to the 6 h time course interval, and returning to near basal levels at the 24 h time course interval. *HSPA* expression gradually decreased at 3 and 6 h post-mortem intervals but, *HSPA* transcripts substantially increased at the 12 h post-mortem interval. At the 24 h post-mortem interval, *HSPA* transcripts were only detectable at low levels, below the initial basal levels observed at 0 h post-mortem 0 h time course interval. A higher level of *HSPA* expression was observed in samples taken at 0 h post-mortem which had been subjected to heat shock, compared to non-heat shocked samples at each time course interval. *HSPA* transcripts

were observed in the 0 h post-mortem interval at 0 h time course, followed by a gradual increase up to the 6 h time course interval, returning back to near basal levels at the 24 h time course interval. At 3 h post-mortem, *HSPA* transcripts were present at high levels at 0 h time course with a gradual decline up to the 24 h time course. *HSPA* expression gradually decreased at 6 h post-mortem but, as with non-heat shock, *HSPA* transcripts substantially increased at 12 h post-mortem. Again, as with non-heat shock, *HSPA* transcripts were only present at low levels at the 24 h post-mortem interval, below or near to the initial basal levels observed at 0 h post-mortem 0 h time course interval.

Furthermore, it is possible to induce the expression of heat shock genes in post mortem tissues, and this expression shows the preconditioning effect characteristic of the heat shock response. This phenomenon, known as thermotolerant preconditioning, is a cellular adaptation resulting from a single severe non-lethal exposure to heat that allows cells to better survive subsequent potentially lethal heat stress episodes, and has been reported previously (Lindquist and Craig, 1988; Moseley, 1997; Pardue *et al*, 2007; Theodorakis and Morimoto, 1987; Theodorakis *et al*, 1999; Tolson and Roberts, 2005). The levels of *HSPA* transcripts in brain tissue that had been subjected to a mild heat shock, the first at 0 h time course followed by subsequent heat shocks at 3, 6 and 24 h time course intervals, showed a marked increase in *HSPA* expression in heat shocked brain tissue compared to non-heat shock within each post-mortem interval. The response of cells to such stress conditions is dependent on the concentration of *HSPA* in the cell prior to stress and on the severity of the stress, based upon its intensity and duration (Lindquist and Craig, 1988; Mosser and Morimoto, 2004; Parsell and Lindquist, 1994).

The results from this study have shown that in the early post-mortem period, (0 h post-mortem), *HSPA* is expressed higher in tissue subjected to single and multiple heat shocks compared to non-heat-shock. However, in later post-mortem intervals, (3 - 24 h post-mortem intervals) results demonstrated some expression however results were inconsistent and somewhat irregular, with no predictive or reproducible patterns. Possible explanation for this could be that there were multiple factors involved in the stress experienced by cells, such as the initial sacrifice procedure by cervical dislocation, the excision of the brain and further damage to the tissues arising from the dissection of the brain. During this period, the cells are also under increased stress from circulatory failure which prevents the delivery of oxygen and removal of waste products of cellular respiration, resulting in anoxia, increased toxin levels and the functional activity of the cells. It is therefore possible that, during this period prior to experimental heat shock, *HSPA* will be up-regulated in response to these cellular changes. This could indicate that the response of the cells subjected to heat shock conditions was dependent on the initial concentration of *HSPA* in the cell prior to heat shock, based on the severity, intensity and duration of stress, (Lindquist and Craig, 1988; Mosser and Morimoto, 2001; Parsell and Lindquist, 1994). Although the production of *HSPA* assists in the protection of cells from the effects of further non-lethal or lethal stresses, they become toxic if they are present in cells for any prolonged period, which would result in cellular death (Theodorakis *et al*, 1999).

Again as expected, *HSPA* protein was detected in both the cytoplasm and the nucleus in all tissue samples at each post-mortem interval for both non-heat shock and heat shocked samples.

It can be concluded from the results of this part of the study that there is *-de novo* synthesis in post mortem brain tissue in response to the changes in cellular conditions during this time. It is possible to induce expression of *HSPA* following systemic death, using an external stimulus, such as heat shock, and that the induction of multiple heat shocks may be suggestive of a preconditioned protection from further stress. However, these initial studies would indicate that *HSPA* would be an unsuitable gene for its use as an early post-mortem marker. Therefore studies in the field of PMI using *HSPA* as an early post-mortem marker requires further investigation. The use of cell lines in replacement for tissue samples would eliminate initial pre stressful events, resulting in low or no detectable levels of *HPSA1A* transcripts. Also, based on the results from the normal and tumourous cell lines, it would be beneficial to include diseased subjects to address these findings.

Chapter 8

References

8.1 References

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8.2 Websites

Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR

http://www6.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf

Dyes and fluorescence detection chemistry in qPCR

www.gene-quantification.com/chemistry.html

DOGS – Database of genome sizes

www.cbs.dtu.dk/databases/DOGS/index.html

European Bioinformatics Institute – ClustalW2, sequence alignment tool

<http://www.ebi.ac.uk/clustalw/>

Gene Cards – human gene database

<http://www.genecards.org>

Gene expression: transcription

www.users.rcm.com/.../T/Transcription.html

NanoDrop 1000 Spectrophotometer V3.7 User's Manual

<http://www.nanodrop.com/Library/nd-1000-v3.7-users-manual-8.5x11.pdf>

National Center for Biotechnology Information

<http://www.ncbi.nlm.nih.gov>

NCBI nucleotide database

<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide&cmd=search&term>

Primer3 – PCR primer design tool

<http://frodo.wi.mit.edu>

The new WHO 2000 classification of brain tumours: imaging correlations

www.rad.usuhs.mil/rad/who/JPA.html

Chapter 9

Appendices

Appendix 9.1 Amino acid and gene sequence for *HSPA1A* *Homo sapiens* adapted from NCBI (<http://www.ncbi.nlm.nih.gov>)

NCBI Reference Sequence: NM_005345.5

Homo sapiens heat shock 70kDa protein 1A (HSPA1A), mRNA

LOCUS NM_005345 2445 bp mRNA linear PRI 20-DEC-2009
DEFINITION Homo sapiens heat shock 70kDa protein 1A (HSPA1A), mRNA.
ACCESSION NM_005345
VERSION NM_005345.5 GI:194248071

Amino Acid Sequence

/translation="MAKAAAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAF
TDTERLIGDAAKNQVALNPQNTVFDKRLIGRKFGDPVVQSDMKHWPQVINDGDKPK
VQVSYKGETKAFYPEEISSMVLTKMKEIAEAYLGYPVTNAVITVPAYFNDSQRQATKD
AGVIAGLNVLRINEPTAAAIAYGLDRTGKGERNVLIFDLGGGTFDVSILTIDDGIFE
VKATAGDTHLGGEDFDNRLVNHFVEEFKRKHKKDISQNKRAVRRLRTACERAKRTLSS
STQASLEIDSLFEGIDFYTSITRARFEELCSDLFRSTLEPVEKALRDAKLDKAQIHDL
VLVGGSTRIPKVQKLLQDFFNGRDLNKSINPDEAVAYGAAVQAAILMGDKSENVQDLL
LLDVAPLSLGLTAGGVMTALIKRNSTIPTKQTQIFTTYSDNQPGVLIQVYEGERAMT
KDNLLGRFELSGIPPAPRGVPQIEVTFDIDANGILNVTATDKSTGKANKITITNDKG
RLSKEEIERMVQEAKEYKAEDVQRERVSAKNALESYAFNMKSAVEDEGLKGKISEAD
KKKVLDKCQEVISWLDANTLAEKDEFEHKRKELEQVCNPIISGLYQGAGGPGPGGFGA

Gene Sequence

1 ataaaagccc aggggcaagc ggtccggata acggctagcc tgaggagctg
ctgcgacagt
61 ccactacctt tttcgagagt gactcccgtt gtcccaaggc ttcccagagc
gaacctgtgc
121 ggctgcaggc accggcgcgt cgagtttccg gcgtccggaa ggaccgagct
cttctcgcg
181 atccagtgtt ccgtttccag cccccaatct cagagcggag ccgacagaga
gcaggggaacc
241 ggcattggcca aagccgcggc gatcggcatc gacctgggca ccacctactc
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 2401 cttcaaagta aataaacttt aaaattcaaa aaaaaaaaaa aaaaa

Appendix 9.2 Amino acid and gene sequence for *HSPA1B* *Homo sapiens* adapted from NCBI (<http://www.ncbi.nlm.nih.gov>)

NCBI Reference Sequence: NM_005346.4

Homo sapiens heat shock 70kDa protein 1B (HSPA1B), mRNA

LOCUS NM_005346 2551 bp mRNA linear PRI 26-SEP-2010
DEFINITION Homo sapiens heat shock 70kDa protein 1B (HSPA1B), mRNA.
ACCESSION NM_005346
VERSION NM_005346.4 GI:167466172

Amino Acid Sequence

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/translation="MAKAAAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAF
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Gene Sequence

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cgtggtgagt
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 ggtgcagaag
 1261 ctgctgcagg acttcttcaa cgggcgcgac ctgaacaaga gcatcaaccc
 cgacgaggct
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Appendix 9.3 Amino acid and gene sequence for *HSPA1L* *Homo sapiens* adapted from NCBI (<http://www.ncbi.nlm.nih.gov>)

NCBI Reference Sequence: NM_005527.3

Homo sapiens heat shock 70kDa protein 1-like (HSPA1L), mRNA

LOCUS NM_005527 2550 bp mRNA linear PRI 20-SEP-2010
DEFINITION Homo sapiens heat shock 70kDa protein 1-like (HSPA1L), mRNA.
ACCESSION NM_005527
VERSION NM_005527.3 GI:124256495

Amino Acid Sequence

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PKVLVSYKGENKAFYPEEISSMVLTKLKETAFAFLGHPVTNAVITVPAYFNDSQRQAT  
KDAGVIAGLNLVRIINEPTAAAIAYGLDKGGQGERHVLIFDLGGGTFDVSILTIDGDI  
FEVKATAGDTHLGGEDFDNRLVSHFVEEFKRKHKKDISQNKRAVRRLRTACERAKRTL  
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DIVLVGGSTRIPKVQRLQLQDYFNDRDLNKSINPDEAVAYGAAVQAAILMGDKSEKVQD  
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MTKDNLLGRFDLTGIPPAPRGVPQIEVTFDIDANGILNVTATDKSTGKVNKITITND  
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SDKNKILDKCNELLSWLEVNQLAEKDEFDHKRKELEQMCNPIITKLYQGGCTGPACGT  
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Gene Sequence

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Appendix 9.4 Amino acid and gene sequence for *HSPA1A Rattus norvegicus* adapted from NCBI (<http://www.ncbi.nlm.nih.gov>)

NCBI Reference Sequence: NM_031971.2

Rattus norvegicus heat shock 70kD protein 1A (Hspa1a), mRNA

LOCUS NM_031971 4045 bp mRNA linear ROD 30-APR-2010
DEFINITION Rattus norvegicus heat shock 70kD protein 1A (Hspa1a), mRNA.
ACCESSION NM_031971
VERSION NM_031971.2 GI:260064044

Amino Acid Sequence

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AGVIAGLNVLRIINEPTAAAIAYGLDRTGKGERNVLIFDLGGGTDFDVSILTIDDGIFE
VKATAGDTHLGGEDFDNRLVSHFVEEFKRKHKKDISQNKRAVRRRLRTACERAKRTLSS
STQASLEIDSLFEGIDFYTSITRARFEELCSDLFRGTLEPVEKALRDAKLDKAQIHDL
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RLSKEEIERMVQEAERYKAEDEVQRERVAAKNALESYAFNMKSAVEDEGLKGKISEAD
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Gene Sequence

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121 actaatgtcc ctcaagagcc caacccatt ttttcttggt tgccaacacc
caaattccaga
181 attaaaactg ggggttcctgt gtggagagcc aagaggagac ctaaggatgg
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 gactctaaca
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 3361 tgggagtgtg tctttgcaat agatataatt tcctgccttc aagtcagcac
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 3421 cccgaagtta tttcttttgc aggacagtca gagctatatt gatatagcaa
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 cacctaaggc
 3661 tgagactctt gttctccttc cctgtataat cttgcctggc cgttgcaact
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 3721 gtgtgaactc ggagatgagt ttacaccacc actgttagtt cacgtttttt
 gtttttacat
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 caaaagaact
 3841 tgccagggtct tgtgtctggt gtcttttgag gtcaggaatt gctgtgtatg
 tcttacagat
 3901 tggactacgc ttgactgaca ttttatggta agatgggggt gggagcacc
 agggactttg
 3961 cattgggatc tgcttgtgtg agaaaaatga aagcagtcag agcaaataac
 aaccagtccg
 4021 gggctgagggt ctagtacagt gcaga
 //

Appendix 9.5 Amino acid and gene sequence for *HSPA1B Rattus norvegicus* adapted from NCBI (<http://www.ncbi.nlm.nih.gov>)

NCBI Reference Sequence: NM_212504.1

Rattus norvegicus heat shock 70kD protein 1B (mapped) (Hspa1b), mRNA

LOCUS NM_212504 5918 bp mRNA linear ROD 20-JUN-2010
DEFINITION Rattus norvegicus heat shock 70kD protein 1B (mapped) (Hspa1b), mRNA.
ACCESSION NM_212504 XM_215309
VERSION NM_212504.1 GI:47059178

Amino Acid Sequence

```
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VQVNYKGENRSFYPEEISSMVLTKMKEIAEAYLGHPVTNAVITVPAYFNDSQRQATKD
AGVIAGLNVLRRIINEPTAAAIAYGLDRTGKGERNVLIFDLGGGTFDVSILTIDDGIFE
VKATAGDTHLGGEDFDNRLVSHFVEEFKRKHKKDISQNKRAVRRLRTACERAKRTLSS
STQASLEIDSLFEGIDFYTSITRARFEELCSDLFRGTLEPVEKALRDAKLDKAQIHDL
VLVGGSTRIPKVQKLLQDFFNDRDLNKSINPDEAVAYGAAVQAAILMGDKSENVQDLL
LLDVAPLSLGLTAGGVMTALIKRNSTIPTKQTQTFTTYSNQPGLIQVYEGERAMT
RDNNLLGRFELSGIPPAPRGVPQIEVTFDIDANGILNVTATDKSTGKANKITITNDKG
RLSKEEIERMVQEAERYKAEDEVQRERVAAKNALESYAFNMKSAVEDEGLKGKISEAD
KKKVLDKCQEVISWLDSENTLAEKEEFVHKREELERVNCNPIISGLYQGAGAPGAGGFGA
QAPKGGSGSGPTIEEVD"
```

Gene Sequence

```
1 gaattccagc acttgggtgt caaggctgga ggagcaagag tttaggcta
gcctgggcta
61 catgagcccc tatTTtgaaa aagaaaaaaa gaaataaaaa agttagaaaa
aagaaaaaat
121 gaaacaaaa gatgtgtgcg tgtgtatgtg tgtgagagag agagacagtc
tctctctctc
181 tctctctctc tctctctctc tctctctctc tctctctctc tgtgtatgtg
tgtgtgtgag
241 acaattactc cctgctgtgg actgttacct cagaactaag actctaagtc
aatctattct
```

301 ttttttaaaa aacaggctcg gggctgggga tttagctcag tggtagagcg
 cttacctagg
 361 aagcgcaagg ccctgggttc ggtccccagc tccgaaaaaa agaaccaaaa
 aaaaaaaaaa
 421 accaaaaaca aaaaaaaaaa acaaaaaaaaa acaaaacaaa acaggctcta
 atgtagattg
 481 ggctagcctc aaactcttca tctcctgcc tccccctcca aagcatgagg
 actacagagg
 541 tggggccacca taccagtaa cctcatactg tttcaagaca tctaactagg
 gggcttttaa
 601 ttcgtacacg gaaggaggag cccggaagaa gaaaaaggga catcactgac
 agctaaacat
 661 atttataatg agaggaggta ggggacagac aagggacgga aaggtgatgt
 ctaggacaaa
 721 aagctaacca cataaactag taggtgggtc ccgacacctc tgcttagggg
 ctcacccaaa
 781 agtcggagtt aacagcactg tgaggccaga tgctatcaga ggacaggaca
 caccatctct
 841 ccacctagga aaggtgaact gacaggggca gcagacacag aagaatcagc
 aaactcttaa
 901 gtcaaccctg aggttactac agcacagaat agaacagtgc ctggcaagga
 gcagctgtgt
 961 gaaggagaag ccaagcagcc atcctcagca ttcttcttct attaattctt
 tgggacaggg
 1021 tctgcctctg taacctaggc tgaccttgac tcgctgcagt ccctctgccc
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 gacactttcc
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 ataaagacag
 1321 caaagcgaat aaactatact gcaagatctc ttctctttcc ctatttaaac
 ctaaaatgga
 1381 gggagtgggg ggcagacaca gacaggcgag cattccacag gcgccccccc
 cacgctgtca
 1441 cttccaggca ggacccaatc acagacttct tagccaagcg ttatccctcc
 cgttttgaga
 1501 aactttctgc gtccgccatc ctgtaggaag aatttgtaca ccttaaactc
 cctccctggt
 1561 ctgattccca aatgtctctc accgcccagc actttcagga gctgaccctt
 ctcagcttca
 1621 catacagaga ccgtacctt gcgtgcccat ggcaacactg tcacaaccgg
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 ccgtctggag
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 agaagctcta
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 cgctgattgg
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 atggccaaga

2161 aaacagcgat cggcatcgac ctgggcacca cctactcgtg cgtgggcgctg
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 tacgtggcct
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 cccaaggtgc
 2461 aggtgaacta caagggcgag aaccggctgt tctaccggga ggagatctcg
 tccatggtgc
 2521 tgaccaagat gaaggagatc gccgaggcgt acctggggcca cccggtgacc
 aacgcggtga
 2581 tcaccgtgcc cgcctacttc aacgactcgc agcggcaggc caccaaggac
 gcgggcgtga
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 5821 atgtaatttt agcatgtcca aacagacacg ttaaggacat cctagggtaa
 agaagtcttc
 5881 agcaatagtt taattgaggc aattatgctc atgaattc

from NCBI (<http://www.ncbi.nlm.nih.gov>)

Rattus norvegicus heat shock protein 1-like (Hspa1l), mRNA

Amino Acid Sequence

Gene Sequence

446

361 acactgtttt tgacgcaaaa cgtctaattg gcaggaagtt taatgatccc
 gtcgtgcagt
 421 cggatatgaa gctgtggcca tttcaagtga tcaatgaagc gggcaagcct
 aaggtgctgg
 481 tgtcctacaa aggagagaag aaagccttct acccgaggga gatctcgtcc
 atggtgctga
 541 ccaagatgaa ggagactgca gaggcctttt tgggccacag tgtcaccaac
 gctgtgatca
 601 ccgtgccagc ctatttcaat gactcccaga gacaggccac taaagatgca
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 661 caggactcaa cgtgctgca atcatcaatg agccacagc ggcggccatc
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 agaaccacg

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tcatccacac
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Appendix 9.7 Amino acid and gene sequence for *GAPDH Homo sapiens* adapted from NCBI (<http://www.ncbi.nlm.nih.gov>)

NCBI Reference Sequence: NM_002046.3

Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA

LOCUS NM_002046 1310 bp mRNA linear PRI 16-MAY-2010
DEFINITION Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH) , mRNA .
ACCESSION NM_002046
VERSION NM_002046.3 GI:83641890

Amino Acid Sequence

/translation="MGKVKVGVNGFGRIGRLVTRAAFNSGKVDIVAINDPFIDLNYMV
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TTMEKAGAHLQGGAKRVIIISAPSADAPMFVMGVNHEKYDNSLKIIISNASCTTNCLAPL
AKVIHDNFGIVEGLMTTVHAITATQKTVDGPGSKLWRDGRGALQNIIPASTGAAKAVG
KVIPELNGKLTGMAFRVPTANVSVVDLTCRLEKPAKYDDIKKVVKQASEGPLKGILGY
TEHQVSSDFNSDTHSSTFDAGAGIALNDHFVKLISWYDNEFGYSNRVVDLMAHMASK

Gene Sequence

1 aaattgagcc cgcagcctcc cgcttcgctc tctgctcctc ctgttcgaca
gtcagccgca
61 tcttcttttg cgtcgccagc cgagccacat cgctcagaca ccatggggaa
ggtgaaggtc
121 ggagtcaacg gatttggtcg tattgggcgc ctggtcacca gggctgcttt
taactctggt
181 aaagtggata ttgttgccat caatgacccc ttcattgacc tcaactacat
ggtttacatg
241 ttccaatatg attccaccca tggcaaattc catggcaccg tcaaggctga
gaacgggaag
301 cttgtcatca atggaaatcc catcaccatc ttccaggagc gagatccctc
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421 aaggctgggg ctcatctgca ggggggagcc aaaaggggtca tcatctctgc
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481 gatgccccca tgttcgtcat ggggtgtgaac catgagaagt atgacaacag
cctcaagatc
541 atcagcaatg cctcctgcac caccaactgc ttagcacccc tggccaagg
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601 aactttggta tcgtggaagg actcatgacc acagtccatg ccatcactgc
caccagaag
661 actgtggatg gcccctccgg gaaactgtgg cgtgatggcc gcggggctct
ccagaacatc

721 atccctgcct ctactggcgc tgccaaggct gtgggcaagg tcatccctga
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 781 aagctcactg gcatggcctt ccgtgtcccc actgccaacg tgtcagtggt
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 gaggggccta
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Appendix 9.8 Amino acid and gene sequence for *GAPDH Rattus norvegicus* adapted from NCBI (<http://www.ncbi.nlm.nih.gov>)

NCBI Reference Sequence: NM_017008.3

Rattus norvegicus glyceraldehyde-3-phosphate dehydrogenase (Gapdh), mRNA

LOCUS NM_017008 1307 bp mRNA linear ROD 16-MAY-2010
DEFINITION Rattus norvegicus glyceraldehyde-3-phosphate dehydrogenase (Gapdh) , mRNA .
ACCESSION NM_017008 XM_216453
VERSION NM_017008.3 GI:110347607

Amino Acid Sequence

/translation="MVKVGVNGFGRIGRLVTRAAFSCDKVDIVAINDPFIDLNYMVYM
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MEKAGAHKGGAKRVIIISAPSADAPMFVMGVNHEKYDNSLKIVSNASCTTNCLAPLAK
VIHDNFGIVEGLMTTVHAITATQKTVDGPGSLWRDGRGAAQNIIPASTGAAKAVGKV
IPELNGKLTGMAFRVPTPNVSVVDLTCRLEKPAKYDDIKKVVKQAAEGPLKGILGYTE
DQVVSCDFNSNSHSSTFDAGAGIALNDNFVKLISWYDNEYGYSNRVVDLMAYMASKE"

Gene Sequence

1 gggggctctc tgctcctccc tggtctagag acagccgcat cttcttgtagc
agtccagcc
61 tcgtctcata gacaagatgg tgaaggctcg tgtgaacgga tttggccgta
tcggacgcct
121 ggttaccagg gctgccttct cttgtgacaa agtggacatt gttgccatca
acgacccctt
181 cattgacctc aactacatgg tctacatggt ccagtatgac tctaccacg
gcaagttcaa
241 cggcacagtc aaggctgaga atgggaagct ggtcatcaac gggaaaccca
tcaccatctt
301 ccaggagcga gatcccgcga acatcaaagt gggatgatgct ggtgctgagt
atgtcgtgga
361 gtctactggc gtcttcacca ccatggagaa ggctggggct cacctgaagg
gtggggccaa
421 aagggtcatc atctccgccc cttccgctga tgcccccattg tttgtgatgg
gtgtgaacca
481 cgagaaatat gacaactccc tcaagattgt cagcaatgca tcctgcacca
ccaactgctt
541 agcccccttg gccaaaggta tccatgacaa ctttggcatc gtggaagggc
tcatgaccac
601 agtccatgcc atcaactgcca ctcagaagac tgtggatggc ccctctggaa
agctgtggcg
661 tgatggccgt ggggcagccc agaacatcat ccctgcatcc actggtgctg
ccaaggctgt

721 gggcaaggtc atcccagagc tgaacgggaa gtcactggc atggccttcc
 gtgttcctac
 781 ccccaatgta tccgttgtag atctgacatg ccgcctggag aaacctgcca
 agtatgatga
 841 catcaagaag gtggtgaagc aggcggccga gggcccacta aaggcatcc
 tgggctacac
 901 tgaggaccag gttgtctcct gtgacttcaa cagcaactcc cattcttcca
 cctttgatgc
 961 tggggctggc attgctctca atgacaactt tgtgaagctc atttcctggt
 atgacaatga
 1021 atatggctac agcaacaggg tggtaggacct catggcctac atggcctcca
 aggagtaaga
 1081 aaccctggac caccagccc agcaaggata ctgagagcaa gagagaggcc
 ctgagttgct
 1141 gaggagtccc catcccaact cagcccccaa cactgagcat ctccctcaca
 attccatccc
 1201 agaccccata acaacaggag gggcctgggg agccctccct tctctcgaat
 accatcaata
 1261 aagttcgctg caccctcaaa aaaaaaaaaa aaaaaaaaaa aaaaaaa

HSPA1A Primer3 Output Homo sapiens

453

(adapted from Primer3).

Using 1-based sequence positions

PRODUCT SIZE: 213, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 3.00

gtggcctacggggcggcggtgcaggcggccatcctgatgggggacaagtccgagaacgtg

caggacctgctgctgctggacgtggctccctgtcgctggggctggagacggccggagggc

gtgatgactgccctgatcaagcgcaactccaccatccccaccaagcagacgcagatttc
<<<<<<<<<<<<<<<<<

KEYS

454

(adapted from Primer3).

Using 1-based sequence positions

PRODUCT SIZE: 156, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 0.00

181 attaaaactggggttcctgtgtggagagccaaggagacctaaggatggtggcttccac
 >>>>>>>>>>>>>>>>>>>

241 tagaccacgtctgtaaatacaatcaaaccctaagaaaattcttggccattaaataagaacc

301 aactggaattccaggctcacctggaatctctacgccttcgatccagtttgaaaaatttga
 <<<<<<<<<<<<<<<<<

+ 3685 nucleotides

Key:

```
>>>>> left (sense)forward primer binding site
<<<<< right(antisense)reverse primer binding site
```

GAPDH Primer3 Output Homo sapiens

(adapted from Primer3)

Using 1-based sequence positions

PRODUCT SIZE: 207, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 0.00

61 tcgtctcatagacaagatggtgaaggctcggtgtgaacggatttggccgtatcggacgcct

121 ggttaccagggctgccttctcttgtgacaaagtggacattgttgccatcaacgacccctt

+ 1067 nucleotides

Key:

```
>>>>> left (sense)forward primer binding site
<<<<< right(antisense)reverse primer binding site
```

Appendix 9.14 Gene Sequence Alignment for *HSPA1A* (1), *HSPA1B* (2) and *HSPA1L*

(3) *Homo sapiens*

| SeqA | NameLen(nt) | SeqB | NameLen(nt) | Score |
|-------|-------------|----------|-------------|-------|
| ===== | | | | |
| 1 | 1 2445 | 2 2 2551 | | 90 |
| 1 | 1 2445 | 3 3 2550 | | 64 |
| 2 | 2 2551 | 3 3 2550 | | 61 |
| ===== | | | | |

```

1      ATAAAAGCCCAGGGGCAAGCGGTCCGGATAACGGCTAGCCTGAGGAGCTGCTGCGACAGT
60
2      -----GGAAAACGGCCAGCCTGAGGAGCTGCTGCGAGGGT
35
3      -----T 1
                                     *

1      CCACTACCTTTTTCGAGAGTGACTCCCGTTGTCCCAGGCTTCCCAGAGCGAACCTGTGC
120
2      CCGCTTCGTCTTTTCGAGAGTGACTCCCGCGGTCCCAGGCTTCCCAGAGCGAACCTGTGC
95
3      TTGGA AAAAGATTACTGAGT---TGGAGCCGTCTCAAATTTGCAGGGAGGGACGGGGTGG
58
                                     **   ****   *   *   ***   **   *   ***   *
1      GGC-TGCAGGCACCGGCGCGTTCGAGTTCCGGCGTCCGGAAGGACCGAGCTCTTCTCGCG
179
2      GGC-TGCAGGCACCGGCGGTGTGAGTTCCGGCGTCCGAAAGACTGAGCTCTTGTTCGCG
154
3      GGGGTGGGGGACCCCGGTTGTGAGTTTGATATTGAGGGAGCCCCACCTACTCGCTGG
118
                                     **   **   **   ***   *   *   **   *   *   **   *   *   *   *
1      GATCCAGTGTTT---CGTTTCCAGCCCCCAATCTCAGAGCGGAGCCGACAGAGAGCAGG
235
2      GATCCCGTCCGC---CGTTTCCAGCCCCAGTCTCAGAGCGGAGCCACAGA--GCAGG
208
3      GGCTGCGTAATCTGGACGTTTCCAAACTGAAGCGAAGGCGTCGGGAGACTAGGCCTCAGA
178
                                     *   **   *   ****   *   *   *   *   *   **   ***
1      GAACC---GGCATGGCCAAAGCCGCGGCGATCGGCATCGACCTGGGCACCACTACTCCT
292
2      GCACC---GGCATGGCCAAAGCCGCGGCGATCGGCATCGACCTGGGCACCACTACTCCT
265
3      GAACCATGGCTACTGCCAAGGGAATCGCCATAGGCATCGACCTGGGCACCACTACTCCT
238
                                     *   ***   *   *   *****   *   **   **   *****
1      GCGTGGGGGTGTTCCAACACGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGCA
352
2      GCGTGGGGGTGTTCCAACACGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGCA
325
3      GTGTGGGGGTGTTCCAGCACGGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGCA
298
                                     *   *****
1      CCACCCCAAGCTACGTGGCCTTCACGGACACCGAGCGGCTCATCGGGGATGCGGCCAAGA
412
2      CCACCCCAAGCTACGTGGCCTTCACGGACACCGAGCGGCTCATCGGGGATGCGGCCAAGA
385
3      CCACCCCAAGCTACGTGGCCTTCACAGACACCGAGCGGCTCATTTGGGGATGCGGCCAAGA
358
                                     *****   *****   *****

```

1 ACCAGGTGGCGCTGAACCCGCAAGAACCCGTGTTTGACGCGAAGCGGCTGATTGGCCGCA
472
2 ACCAGGTGGCGCTGAACCCGCAAGAACCCGTGTTTGACGCGAAGCGGCTGATCGGCCGCA
445
3 ACCAGGTAGCAATGAATCCCCAGAACACTGTTTTTGATGCTAAACGTCTGATCGGCAGGA
418
***** ** ***** ** ***** ** ***** ** ***** ** *

1 AGTTCGGCGACCCGGTGGTGCACTCGGACATGAAGCACTGGCCTTTCCAGGTGATCAACG
532
2 AGTTCGGCGACCCGGTGGTGCACTCGGACATGAAGCACTGGCCTTTCCAGGTGATCAACG
505
3 AATTTAATGATCCTGTGTACAAGCAGATATGAACTTTGGCCTTTTCAAGTGATTAATG
478
* ** **

1 ACGGAGACAAGCCCAAGGTGCAGGTGAGCTACAAGGGGGAGACCAAGGCATTCTACCCCG
592
2 ACGGAGACAAGCCCAAGGTGCAGGTGAGCTACAAGGGGGAGACCAAGGCATTCTACCCCG
565
3 AAGGAGGCAAGCCCAAAGTCCTTGTGTCTACAAGGGGGAGAATAAAGCTTTCTACCCCTG
538
* ***** ** * * * * * * * * * * * * * * * * *

1 AGGAGATCTCGTCCATGGTGCTGACCAAGATGAAGGAGATCGCCGAGGCGTACCTGGGCT
652
2 AGGAGATCTCGTCCATGGTGCTGACCAAGATGAAGGAGATCGCCGAGGCGTACCTGGGCT
625
3 AGGAAATCTCTTCGATGGTATTGACTAAGTTGAAGGAGACTGCTGAGGCCTTTTGGGCC
598
***** ***** ** ***** ***** ** ***** * *****

1 ACCCGGTGACCAACGCGGTGATCACCGTGCCGGCCTACTTCAACGACTCGCAGCGCCAGG
712
2 ACCCGGTGACCAACGCGGTGATCACCGTGCCGGCCTACTTCAACGACTCGCAGCGCCAGG
685
3 ACCCTGTACCAATGCAGTGATTACCGTGCCAGCCTATTTCAATGACTCTCAACGTCAGG
658
***** ** ***** ** ***** ***** ***** ***** ***** ** * * * *

1 CCACCAAGGATGCGGGTGTGATCGCGGGGCTCAACGTGCTGCGGATCATCAACGAGCCCA
772
2 CCACCAAGGATGCGGGTGTGATCGCGGGGCTCAACGTGCTGCGGATCATCAACGAGCCCA
745
3 CTACTAAGGATGCAGGTGTGATTGCTGGACTTAATGTGCTAAGAAATCATCAATGAGCCCA
718
* ** ***** ***** ** * * * * * * * * * * * * * * * * *

1 CGGCCCGCCCATCGCCTACGGCCTGGACAGAACGGGCAAGGGGGAGCGCAACGTGCTCA
832
2 CGGCCCGCCCATCGCCTACGGCCTGGACAGAACGGGCAAGGGGGAGCGCAACGTGCTCA
805
3 CGGCTGCTGCCATTGCCTATGGTTTAGATAAAGGAGGTCAAGGAGAACGACATGTCCTGA
778
***** ** ***** ***** * * * * * * * * * * * * * * * *

1 TCTTTGACCTGGGCGGGGGACCTTCGACGTGTCCATCCTGACGATCGACGACGGCATCT
892
2 TCTTTGACCTGGGCGGGGGACCTTCGACGTGTCCATCCTGACGATCGACGACGGCATCT
865
3 TTTTGTATCTGGGTGGAGGCACATTTGATGTGTCAATTCTGACCATAGATGATGGGATTT
838
* ***** ***** ** ***** ** * * * * * * * * * * * * * * *

1 TCGAGGTGAAGGCCACGGCCGGGGACACCCACCTGGGTGGGGAGGACTTTGACAACAGGC
952
2 TCGAGGTGAAGGCCACGGCCGGGGACACCCACCTGGGTGGGGAGGACTTTGACAACAGGC
925
3 TTGAGGTAAAGGCCACTGCTGGGGACACTCACCTGGGTGGGGAGGACTTTGACAACAGGC
898

```

* * * * *
1      TGGTGAACCACTTCGTGGAGGAGTTCAAGAGAAAACACAAGAAGGACATCAGCCAGAACA
1012
2      TGGTGAACCACTTCGTGGAGGAGTTCAAGAGAAAACACAAGAAGGACATCAGCCAGAACA
985
3      TTGTGAGCCACTTCGTGGAGGAGTTCAAGAGGAAACACAAAAAGGACATCAGCCAGAACA
958
* * * * *

1      AGCGAGCCGTGAGGCGGCTGCGCACCGCCTGCGAGAGGGCCAAGAGGACCCTGTCGTCCA
1072
2      AGCGAGCCGTGAGGCGGCTGCGCACCGCCTGCGAGAGGGCCAAGAGGACCCTGTCGTCCA
1045
3      AGCGAGCCGTGAGGCGGCTGCGCACCGCCTGCGAGAGGGCCAAGAGGACCCTGTCGTCCA
1018
* * * * *

1      GCACCCAGGCCAGCCTGGAGATCGACTCCCTGTTTGAGGGCATCGACTTCTACACGTCCA
1132
2      GCACCCAGGCCAGCCTGGAGATCGACTCCCTGTTTGAGGGCATCGACTTCTACACGTCCA
1105
3      GCACCCAGGCCAACCTAGAAATTGATTCACTTTATGAAGGCATTGACTTCTATACATCCA
1078
* * * * *

1      TCACCAGGGCGAGGTTTCGAGGAGCTGTGCTCCGACCTGTTCCGAAGCACCTGGAGCCCG
1192
2      TCACCAGGGCGAGGTTTCGAGGAGCTGTGCTCCGACCTGTTCCGAAGCACCTGGAGCCCG
1165
3      TCACCAGAGCTCGATTTGAAGAGTTGTGTGCAGACCTGTTTAGGGGTACCCTGGAGCCTG
1138
* * * * *

1      TGGAGAAGGCTCTGCGCGACGCCAAGCTGGACAAGGCCAGATTACGACCTGGTCCTGG
1252
2      TGGAGAAGGCTCTGCGCGACGCCAAGCTGGACAAGGCCAGATTACGACCTGGTCCTGG
1225
3      TAGAAAAAGCGCTTCGGGATGCCAAGATGGATAAAGCTAAAAATCCATGACATTGTTTTAG
1198
* * * * *

1      TCGGGGGCTCCACCCGCATCCCCAAGGTGCAGAAGCTGCTGCAGGACTTCTTCAACGGGC
1312
2      TCGGGGGCTCCACCCGCATCCCCAAGGTGCAGAAGCTGCTGCAGGACTTCTTCAACGGGC
1285
3      TAGGGGGCTCCACCCGCATCCCCAAGGTGCAGCGGCTGCTTCAGGACTACTTCAATGGAC
1258
* * * * *

1      GCGACCTGAACAAGAGCATCAACCCCGACGAGGCTGTGGCCTACGGGGCGGCGGTGCAGG
1372
2      GCGACCTGAACAAGAGCATCAACCCCGACGAGGCTGTGGCCTACGGGGCGGCGGTGCAGG
1345
3      GTGATCTCAACAAGAGCATCAACCTGATGAGGCCGTAGCATATGGGGCTGCGGTACAAG
1318
* * * * *

1      CGGCCATCCTGATGGGGGACAAGTCCGAGAACGTGCAGGACCTGCTGCTGCTGGACGTGG
1432
2      CGGCCATCCTGATGGGGGACAAGTCCGAGAACGTGCAGGACCTGCTGCTGCTGGACGTGG
1405
3      CAGCCATCCTGATGGGGGACAAGTCTGAGAAGGTACAGGACCTGCTGCTGCTGGACGTGG
1378
* * * * *

1      CTCCCCTGTCGCTGGGGCTGGAGACGGCCGGAGGCGTGATGACTGCCCTGATCAAGCGCA
1492
2      CTCCCCTGTCGCTGGGGCTGGAGACGGCCGGAGGCGTGATGACTGCCCTGATCAAGCGCA
1465

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3 CTCCCCTGTCCCTGGGGCTGGAGACGGCTGGGGGCGTGATGACTGCCCTGATAAAGCGCA
1438

1 ACTCCACCATCCCCACCAAGCAGACGCAGATCTTCAACCTACTCCGACAACCAACCCG
1552
2 ACTCCACCATCCCCACCAAGCAGACGCAGATCTTCAACCTACTCCGACAACCAACCCG
1525
3 ACTCCACCATCCCCACCAAGCAGACACAGATTTTCAACCTACTCTGACAACCAACCCG
1498

1 GGGTGCTGATCCAGGTGTACGAGGGCGAGAGGGCCATGACGAAAGACAACAATCTGTTGG
1612
2 GGGTGCTGATCCAGGTGTACGAGGGCGAGAGGGCCATGACGAAAGACAACAATCTGTTGG
1585
3 GGGTGCTGATCCAGGTGTATGAGGGCGAGAGGGCCATGACAAAGACAACAACCTGCTGG
1558

1 GGCGCTTCGAGCTGAGCGGCATCCCTCCGGCCCCAGGGGCGTGCCCCAGATCGAGGTGA
1672
2 GGCGCTTCGAGCTGAGCGGCATCCCTCCGGCCCCAGGGGCGTGCCCCAGATCGAGGTGA
1645
3 GGCGGTTTGACCTGACTGGAATCCCTCCAGCACCCAGGGGAGTTCTCAGATCGAGGTGA
1618

1 CCTTCGACATCGATGCCAACGGCATCCTGAACGTCACGGCCACGGACAAGAGCACCGGCA
1732
2 CCTTCGACATCGATGCCAACGGCATCCTGAACGTCACGGCCACGGACAAGAGCACCGGCA
1705
3 CGTTTGACATTGATGCCAATGGTATTCTCAATGTACAGCCACGGACAAGAGCACCGGCA
1678
* * * * *

1 AGGCCAACAGATCACCATCACCAACGACAAGGGCCGCCTGAGCAAGGAGGAGATCGAGC
1792
2 AGGCCAACAGATCACCATCACCAACGACAAGGGCCGCCTGAGCAAGGAGGAGATCGAGC
1765
3 AGGTGAACAAGATCACCATCACCAATGACAAGGGCCGCCTGAGCAAGGAGGAGATTGAGC
1738

1 GCATGGTGCAGGAGGCGGAGAAGTACAAAGCGGAGGACGAGGTGCAGCGCGAGAGGGTGT
1852
2 GCATGGTGCAGGAGGCGGAGAAGTACAAAGCGGAGGACGAGGTGCAGCGCGAGAGGGTGT
1825
3 GCATGGTTCTGGATGCTGAGAAATATAAAGCTGAAGATGAGGTCCAGAGGGAGAAAATTG
1798

1 CAGCCAAGAACGCCCTGGAGTCCTACGCCCTTCAACATGAAGAGCGCCGTGGAGGATGAGG
1912
2 CAGCCAAGAACGCCCTGGAGTCCTACGCCCTTCAACATGAAGAGCGCCGTGGAGGATGAGG
1885
3 CTGCAAAGAAATGCCTTAGAATCCTATGCTTTTAAACATGAAGAGTGTGTGATGATGAAG
1858
* * * * *

1 GGCTCAAGGGCAAGATCAGCGAGGCGGACAAGAAGAAGGTGCTGGACAAGTGTCAGAGG
1972
2 GGCTCAAGGGCAAGATCAGCGAGGCGGACAAGAAGAAGGTTCTGGACAAGTGTCAGAGG
1945
3 GTTTGAAGGGCAAGATTAGTGAGTCTGATAAAAAATAAATATTGGATAAATGCAACGAGC
1918
* * * * *

1 TCATCTCGTGGCTGGACGCCAACACCTTGGCCGAGAAGGACGAGTTTGAGCACAAGAGGA
2032

462

```

1          AAAAAAAAAA-----
2445
2          CACTACAAAGGCTGGGAATGTATGTTTTTATAATTGTTTATTTAAATATGAAAAATAAA
2508
3          ATGTACCTTGAAGGGGAGAGTTCATGGAGAGAGCATGTGTTCTCTGATTGTGAGGTCACT
2507

```

*

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1          -----
2          ATGTTAACTTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 25513
GTGAATGATTAAATTGGTAAGGGTAAAGTAAAAAAAAAAAAA 2550

```

KEY

- *|| Nucleotides are identical in all sequences in the alignment
- .|| Semi-conserved substitutions are observed
- :|| Conserved substitutions are observed

Appendix 9.15 Amino Acid Sequence Alignment for *HSPA1A* (1), *HSPA1B* (2) and *HSPA1L* (3) *Homo sapiens*

| SeqA | NameLen (aa) | SeqB | NameLen (aa) | Score | | |
|-------|---|------|--------------|-------|-----|-----|
| ===== | | | | | | |
| 1 | 1 | 624 | 2 | 2 | 641 | 100 |
| 1 | 1 | 624 | 3 | 3 | 641 | 90 |
| 2 | 2 | 641 | 3 | 3 | 641 | 89 |
| ===== | | | | | | |
| 1 | --MAKAAAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQ | | | | | |
| 58 | | | | | | |
| 2 | --MAKAAAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQ | | | | | |
| 58 | | | | | | |
| 3 | MATAKGIAIGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQ | | | | | |
| 60 | | | | | | |
| | * * . ***** | | | | | |
| 1 | VALNPQNTVFDAKRLIGRKFGDPVVQSDMKHWPQVINDGDKPKVQVSYKGETKAFYPEE | | | | | |
| 118 | | | | | | |
| 2 | VALNPQNTVFDAKRLIGRKFGDPVVQSDMKHWPQVINDGDKPKVQVSYKGETKAFYPEE | | | | | |
| 118 | | | | | | |
| 3 | VAMNPQNTVFDAKRLIGRKFNDPVVQADMKLWPFQVINEGGKPKVLVSYKGENKAFYPEE | | | | | |
| 120 | | | | | | |
| | ** : ***** . ***** : ** ***** : * . ***** ***** . ***** | | | | | |
| 1 | ISSMVLTKMKEIAEAYLGYPTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRINIPTA | | | | | |
| 178 | | | | | | |
| 2 | ISSMVLTKMKEIAEAYLGYPTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRINIPTA | | | | | |
| 178 | | | | | | |
| 3 | ISSMVLTKLKETAFAFLGHPVTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRINIPTA | | | | | |
| 180 | | | | | | |
| | ***** : ** *** : ** : ***** | | | | | |
| 1 | AAIAYGLDRTGKGERNVLIFDLGGGTFDVSILTIIDDGIFEVKATAGDTHLGGEDFDNRLV | | | | | |
| 238 | | | | | | |
| 2 | AAIAYGLDRTGKGERNVLIFDLGGGTFDVSILTIIDDGIFEVKATAGDTHLGGEDFDNRLV | | | | | |
| 238 | | | | | | |
| 3 | AAIAYGLDKGGQGERHVLIFDLGGGTFDVSILTIIDDGIFEVKATAGDTHLGGEDFDNRLV | | | | | |
| 240 | | | | | | |
| | ***** : * : *** : ***** | | | | | |
| 1 | NHFVEEFKRKHKKDISQNKRAVRRLRTACERAKRTLSSSTQASLEIDSLFEGIDFYTSIT | | | | | |
| 298 | | | | | | |
| 2 | NHFVEEFKRKHKKDISQNKRAVRRLRTACERAKRTLSSSTQASLEIDSLFEGIDFYTSIT | | | | | |
| 298 | | | | | | |
| 3 | SHFVEEFKRKHKKDISQNKRAVRRLRTACERAKRTLSSSTQANLEIDSLYEGIDFYTSIT | | | | | |
| 300 | | | | | | |
| | . ***** . ***** : ***** | | | | | |
| 1 | RARFEELCSDLFRSTLEPVEKALRDAKLDKAQIHDVLVVGSTRIPKVQKLLQDFFNGRD | | | | | |
| 358 | | | | | | |
| 2 | RARFEELCSDLFRSTLEPVEKALRDAKLDKAQIHDVLVVGSTRIPKVQKLLQDFFNGRD | | | | | |
| 358 | | | | | | |
| 3 | RARFEELCADLFRGTLPEPVEKALRDAKMDKAKIHDIVLVGGSTRIPKVQRLLDQYFNGRD | | | | | |
| 360 | | | | | | |
| | ***** : *** . ***** : ** : *** : ***** : *** : ***** | | | | | |
| 1 | LNKSI NPDEAVAYGAAVQAAILMGDKSENVQDLLLLDVAPLSLGLETAGGVMTALIKRNS | | | | | |
| 418 | | | | | | |
| 2 | LNKSI NPDEAVAYGAAVQAAILMGDKSENVQDLLLLDVAPLSLGLETAGGVMTALIKRNS | | | | | |
| 418 | | | | | | |
| 3 | LNKSI NPDEAVAYGAAVQAAILMGDKSEKVQDLLLLDVAPLSLGLETAGGVMTALIKRNS | | | | | |
| 420 | | | | | | |
| | ***** . ***** | | | | | |

norvegicus

466

2 ATTTATAATGAGAGGAGGTAGGGGACAGACAAGGGACGGAAAGGTGATGTCTAGGACAAA 720
3 -----

1 -----CCACTAGACCACGTCTGTAATCAA-----TCAAACCTAAG 272
2 AAGCTAACCACATAAACTAGTAGGTGGGTCCCGACACCTCTGCTTAGGGTCTCACCCTAAA 780
3 -----

1 A-----AAATTCT 280
2 AGTCGGAGTTAACAGCACTGTGAGGCCAGATGCTATCAGAGGACAGGACACACCATCTCT 840
3 -----

1 TGGCCCATTAATAAGAACCAACTGGAATTCCAGGCTCA-----CCTGGAATCTCTACG 334
2 CCACCTAGGAAAGGTGAAGTACAGGGGCAGCAGACACAGAAGAATCAGCAAACCTCTTAA 900
3 -----

1 -----CCTTCGATCCAGTTTGGAAAAATTGAAGTCGCTGAGCCCCTACGA 379
2 GTCAACCCCTGAGGTTACTACAGCACAGAATAGAACAGTGCCTGGCAAGGAGCAGCTGTGT 960
3 -----

1 GTGGGGAGCTCCAGGAA--CATCCAA----- 404
2 GAAGGAGAAGCCAAAGCAGCCATCCTCAGCATTCTTCTTCTATTAATCTTTTGGGACAGGG 1020
3 -----

1 -----CTGAGCAACTGGGGTT-----CCCCCACCCCCACCCCGC 440
2 TCTGCCTCTGTAACTAGGCTGACCTTGACTCGCTGCAGTCCCTCTGCCCCACCTCTAC 1080
3 -----

1 C-----CCTCCCG-----CAA 452
2 CGAAAGTTTTTTTTTTCTCCTTTCTAATGCCAGGCTGCCGCCTGAGGCAAGGGAGCGG 1140
3 -----

1 CTTTGAGCCT-----CTT-----CTGGGACAGATCCT 479
2 CTTGCAGCCTGGCACGGTCGGTCGATTAAACCTTGCTCTCCCGTCCTGGGACACTTTC 1200
3 -----

1 CTGATTCCTAAATAAGTCC-----ATGAGGTCAGAGT--TAGCACTG----- 519
2 TTTTGTGGGTACAGGTCCTCCTAACATGAGAACCGAGTGTTTACACAATGATGTTCTTT 1260
3 -----

1 -----CCATTGTAAACGCGGCTGGAGGG----- 542
2 TGAAACCGTGAAACTCCACAGGCGATGTACTTGTACGTTAAGCGTGACATAAAGACAG 1320
3 -----

1 -----GGTCACGTAC----- 553
2 CAAAGCGAATAAACTATACTGCAAGATCTCTTCTTTCCCTATTTAAACCTAAAAATGGA 1380
3 -----

1 -----CGGACACG-----CCCCAGGCACCTCCCTTGGGTCTCCC 588
2 GGGAGTGGGGGGCAGACACAGACAGGCGAGCATTCACAGGCGCCCCCCCCACG-CTGTC 1439
3 -----

1 AAGGCTAGGAAGGGGAAGTTATAACCTTAACT--CGAGC-----CCCATAATCAG 637
2 ACTTCCAGGCAGGACCCAATCACAGACTTCTTAGCCAAGCGTTATCCCTCCCGTTTGTAG 1499
3 -----

1 -AACTGTGCGAGTCTGCGAACC-----CCCACA 664

2 AAAC TTTCTGCGTCCGCCATCCTGTAGGAAGAA TTTGTACACCTTAAACTCCCTCCCTGG 1559
3 -----

1 AATCACAACCAACTGTC--CACAAACGAGCTAGCAG---TGACCTTTCCTGTCCAT 717
2 TCTGATTCCCAAATGTCTCTACCGCCAGCATTTCAGGAGCTGACCTTCTCAGCTTC 1619
3 -----

1 TCC-ACTCAGGCCTCAGTAATGCGTCGCCATAGCAACAGTGTCAACAGCAGCACCAGCAG 776
2 ACATACAGAGACCGCTACCTTGCGTCGCCATGGCAACACTGTCAACACCGGAACAGCAC 1679
3 -----

1 GTCCCGCCACCTCCCCACAGGAATCCGTCTTTCCAGCTAACCCAGATCTGTCTGGA 836
2 TTCTTACCACCCCGCCTCAGGAATCCAATCTGTCCAGCGAAGCCAGATCCGTCTGGA 1739
3 -----

1 GAGTTCTGAACAGGGGCGGAACCTCAACTCCTATTACTCAAAGGAGGCGGGGAAGCTCC 896
2 GAGTTCTGGACAAGGGCGGTACCTCAACATGGATTACTCATGGAGGCGGAGAAGCTCT 1799
3 ---CAGAAGAGG-----CTCAAAGTTGCCGG----- 23
* : * * * *

1 ACCCGACGCGAAACTGCTGGAAGATTCTTGCCCCAAGGCCTCCTCCCGCTCGCTGATTG 956
2 AACAGACCCGAAACTGCTGGAAGATTCTTGCCCCAAGGCCTCCTCCCGCTCGCTGATTG 1859
3 -----

1 GCCCATGGGAGGGTGGGCGGGGCCGAGGAGGCTCCTTAAAGGCGCAGGGCGGCGCGCAG 1016
2 GCCCATGGGAGGGTGGGCGGGGCCGAGGAGGCTCCTTAAAGGCGCAGGGCGGCGCGCAG 1919
3 -----GAGG-----GGCGGGTGGGGGTG-----GGGG-----G 48
* * * * * * * * * * * * * * *

1 GACACCAGATTCTCTCTTAATCTGACAGAATCAGTTTCTGGTTCCACTCGCAGAGAAG 1076
2 GACACCAGATTCTCTCTTAATCTGACAGAATCAGTTTCTGGTTCCACTCGCAGAGAAG 1979
3 AATCCAGTTG-----CTCAGTTTGGATGTTCTCGGAGCT---CCCCACTCGTAGGGG 98
* * * * * * * * * * * * * * *

1 CAGAGAAGCGGAGCAAGCGGCGCTTCCAGAACCTCGGGCAAGACCAGCCTCTCCCAGAG 1136
2 CAGAGAAGCGGAGCAAGCGGCGCTTCCAGAACCTCGGGCAAGACCAGCCTCTCCCAGAG 2039
3 CT-----CAGCGACTTCAAAATTTTCCAACTGGATCG----- 130
* : * * * * * * * * * * * * *

1 CATCCCCACCGCGAAGCGCAACCTTCTCCAGAGCATCCCCAGCGGAGCGCACCTTCCC 1196
2 CATCCCCACCGCGAAGCGCAACCTTCTCCAGAGCATCCCCAGCGGAGCGCACCTTCCC 2099
3 -----AAGGCGTAGAGATTCCAGG-----CCT 152
* * * * * * * * * * *

1 CAGAGCATCCCCGCCGCCAAGCGCAACCTTCCAGAAGCAGAGAGCGGCGCATGAGCCAA 1256
2 CAGAGCATCCCCGCCGCCAAGCGCAACCTTCCAGAAGCAGAGAGCGGCGCATGAGCCAA 2159
3 CGGAAAACCATGGCTGCTAA-----TAAA 176
* * * * * * * * * *

1 AAAACAGCGATCGGCATCGACCTGGGCAACACCTACTCGTGCCTGGGCGTGTTCAGCAC 1316
2 AAAACAGCGATCGGCATCGACCTGGGCAACACCTACTCGTGCCTGGGCGTGTTCAGCAC 2219
3 GGAATGGCCATAGGCATCGACCTGGGCAACACCTACTCGTGCCTGGGCGTGTTCAGCAC 236
* * * * * * * * * *

1 GGC AAGGTGGAGATCATCGCCAACGACCAAGGGCAACCGCACGACCCCCAGCTACGTGGCC 1376
2 GGC AAGGTGGAGATCATCGCCAACGACCAAGGGCAACCGCACGACCCCCAGCTACGTGGCC 2279
3 GGC AAGGTGGAGATCATCGCCAACGACCAAGGGCAACCGCACGACCCCCAGCTACGTGGCC 296
* * * * * * * * * *

1 TTCACCGACACCGAGCGGCTCATCGGGGACGCCGCCAAGAACAGGTGGCGCTGAACCCG 1436
2 TTCACCGACACCGAGCGGCTCATCGGGGACGCCGCCAAGAACAGGTGGCGCTGAACCCG 2339
3 TTCACCGACACCGAGCGGCTCATCGGGGACGCCGCCAAGAACAGGTGGCCATGAATCCC 356
* * * * * * * * * *

1 CAGAACAACCGTGTTCGACGCGAAGCGGCTGATCGGCCGCAAGTTTCGGCGACCCGGTGGTG 1496

2 CAGAAACACCGTGTTCGACGCGAAGCGGCTGATCGGCCGCAAGTTCGGCGACCCGGTGGTG 2399
3 CAGAACTGTGTTTTTGACGCCAAACGTCTAATTGGCAGGAAGTTTAAATGATCCCGTCGTG 416
***** ** ** ***** ** . ** . ** * * * * . * * * * * *

1 CAGTCGGACATGAAGCACTGGCCCTTCCAGGTGGTGAACGACGGCGACAAGCCCAGGGTG 1556
2 CAGTCGGACATGAAGCACTGGCCCTTCCAGGTGGTGAACGACGGCGACAAGCCCAGGGTG 2459
3 CAGTCGGATATGAAGCTGTGGCCATTTCAGTGATCAATGAAGCGGGCAAGCCTAAGGTG 476
***** : ***** . * * . * * . * * * * . * * * * * *

1 CAGGTGAAGTACAAAGGCGAGAACCGGTGCTTCTACCCGGAGGAGATCTCGTCCATGGTG 1616
2 CAGGTGAAGTACAAAGGCGAGAACCGGTGCTTCTACCCGGAGGAGATCTCGTCCATGGTG 2519
3 CTGGTGTCTTACAAAGGAGAGAAAGACCTTCTACCCGGAGGAGATCTCGTCCATGGTG 536
* : * * * * . : * * * * . * * . * * * * . . . * * * * * * * * * * * * *

1 CTGACCAAGATGAAGGAGATCGCCGAGGCGTACCTGGGCCACCCGGTGACCAACGCGGTG 1676
2 CTGACCAAGATGAAGGAGATCGCCGAGGCGTACCTGGGCCACCCGGTGACCAACGCGGTG 2579
3 CTGACCAAGATGAAGGAGACTGACAGGCGTTTTTTGGGCCACAGTGTCACCAACGCTGTG 596
* * * * * * * * * * * * * * * * * * . * * . * * * * * : * * * * * * * * * * * * *

1 ATCACCGTGCCCGCCTACTTCAACGACTCGCAGCGGCAGGCCACCAAGGACGCGGGCGTG 1736
2 ATCACCGTGCCCGCCTACTTCAACGACTCGCAGCGGCAGGCCACCAAGGACGCGGGCGTG 2639
3 ATCACCGTGCCAGCCTATTTCATGACTCCAGAGACAGGCCACTAAGATGACAGGGGTC 656
* * * * * * * * * * . *

1 ATCGCGGGTCTGAACGTGCTGCGGATCATCAACGAGCCACGGCGGCCCGCCATCGCCTAC 1796
2 ATCGCGGGTCTGAACGTGCTGCGGATCATCAACGAGCCACGGCGGCCCGCCATCGCCTAC 2699
3 ATCGCAGGACTCAACGTGCTGCGAATCATCAATGAGCCACAGCGCGGCCCGCCATCGCCTAC 716
* * * * * . * * : *

1 GGGCTGGACCGGACCGGCAAGGGCGAGCGCAACGTGCTCATCTTCGACCTGGGGGGCGGC 1856
2 GGGCTGGACCGGACCGGCAAGGGCGAGCGCAACGTGCTCATCTTCGACCTGGGGGGCGGC 2759
3 GGCTTGGATAAAGGAAGTCACGGAGAGCGGCACGTGCTCATCTTCGACCTGGGGGGCGGC 776
* * * * * * * * * * * . * * * . * * * * * . * * * * * * * * * * * * *

1 ACGTTCGACGTGTCCATCCTGACGATCGACGACGGCATCTTCGAGGTGAAGGCCACGGCG 1916
2 ACGTTCGACGTGTCCATCCTGACGATCGACGACGGCATCTTCGAGGTGAAGGCCACGGCG 2819
3 ACGTTCGACGTGTCCATCCTGACGATCGACGACGGCATCTTCGAGGTGAAGGCCACGGCG 836
* *

1 GGCGACACGCACCTGGGCGGGGAGGACTTCGACAACCGGCTGGTGAGCCACTTCGTGGAG 1976
2 GGCGACACGCACCTGGGCGGGGAGGACTTCGACAACCGGCTGGTGAGCCACTTCGTGGAG 2879
3 GGCGACACGCACCTGGGCGGGGAGGACTTCGACAACCGGCTGGTGAGCCACTTCGTGGAG 896
* *

1 GAGTTCAGAGGAAGCACAAGAAGGACATCAGCCAGAACAGCGCGCGGTGCGGCGACTG 2036
2 GAGTTCAGAGGAAGCACAAGAAGGACATCAGCCAGAACAGCGCGCGGTGCGGCGACTG 2939
3 GAGTTCAGAGGAAGCACAAGAAGGACATCAGCCAGAACAGCGCGCGGTGCGGCGTCTG 956
* *

1 CGCACGGCGTGCGAGAGGGCCAAGAGGACGCTGTCTGTCAGCACCCAGGCCAGCCTGGAG 2096
2 CGCACGGCGTGCGAGAGGGCCAAGAGGACGCTGTCTGTCAGCACCCAGGCCAGCCTGGAG 2999
3 CGCACGGCGTGCGAGAGGGCCAAGAGGACGCTGTCTGTCAGCACCCAGGCCAACCTGGAG 1016
* *

1 ATCGACTCTCTGTTCGAGGGCATCGACTTCTACACGTCCATCACGCGGGCGCGGTTCGAG 2156
2 ATCGACTCTCTGTTCGAGGGCATCGACTTCTACACGTCCATCACGCGGGCGCGGTTCGAG 3059
3 ATCGACTCTCTGTACGAGGGCATCGACTTCTACACGTCCATCACGCGGGCGCGGTTCGAG 1076
* * * * * * * * * * : *

1 GAGCTGTGCTCGGACCTGTTCCGCGGCACGCTGGAGCCCGTGGAGAAGGCCCTGCGCGAC 2216
2 GAGCTGTGCTCGGACCTGTTCCGCGGCACGCTGGAGCCCGTGGAGAAGGCCCTGCGCGAC 3119
3 GAGCTGTGTCAGACCTATTTAGAGGCACGCTTGAGCCCGTGGAGAAGTCTCTCCGGGAC 1136
* * * * * * * * . * * * * * . * * . * . * * * * * * * * * * * * * * * * * *

1 GCCAAGCTGGACAAAGCGCAGATCCACGACCTGGTGTGTTGGGCGGCTCGACGCGCATC 2276
2 GCCAAGCTGGACAAAGCGCAGATCCACGACCTGGTGTGTTGGGCGGCTCGACGCGCATC 3179
3 GCCAAGATGGACAAGGCTAAAATCCATGACATTGTTCTAGTAGGGGCTCTACCCGCATC 1196
* * * * * . * * * * * * * * * * . * * * * * * * * . * * * * * * * * * * * * *

1 CCCAAGGTGACAGAGCTGCTGCAGGACTTCTTCAACGGGCGCGACCTGAACAAGAGCATC 2336

470

2 GCCCAGGCGCCCCAAGGGAGGGCTCTGGGTCGGGGCCCCACCATCGAGGAGGTGGATTAGAGG 4079
3 CCAGGGTATACACCCGGCAGGGCTGCCACAGGCCCCACCATTGAGGAAGTAGATTA--GC 2088
* . * . . * . . . * * . * * * : * * * * * * * * * * * * * * * *

1 CTCTTTCTGGCGCTCCAGGTGTGATCTAGGAGACAGATGGGTGG--CCTTGAGGACTTTGG 3235
2 CT-TTTCTGGCTCTCAGGGTGTGGCTAG-AGACAGACTCTTGATGGCTGCTGGTGCACG 4137
3 CTTTCCCAGAAAGGCAGGGTG---CTAG-----GGTGCCCTCTAGGCG-ACA 2130
* * * * * : * . . * * * * * * * * * * * * * * * * * * : .

1 GTTATTGTCTGTTTAGGACATTAACTCCTTCGTTCTG-----GTCTGCAATCAA 3282
2 ATTCTTATCAAGTTACTCCTTCTCTCCGGAGTTCAGTTTAAAGTTACAGCCTTTTATACG 4197
3 TTTATTACCT---CCCAACATCACTGTGATTCT-----TGAAGTACTG 2172
* * . * * : * : * . : . * * * * * * * * * * : : . : . .

1 GTCCTAGGTTTAAAGCAAACTGCCTTCCATTTACTCTGTGGAATTTACGTTGTCT---- 3337
2 GTAATTGATTTGAGTTTGTACATTTTGTATGCTCGTGGGTTTTTATATATTCAAATTA 4257
3 GACTTGAGCCTAAGTCACCATCCTTTGG--GATCTG----- 2206
* . * . . * . * * : : * . * * . . . * *

1 --TTGCATTCCCAGTAAATTAGTACTGGGAGTGTGTCTTTGCAATAGATATAATTTCCCT 3394
2 AGGTGTCATGTTCTTTGCGTTTAACTAAGTAGCTGTG-TAAAAATGGTGTTCCTTCCT 4316
3 --ATGCAG-----AACCCACG 2220
: * * * * : . . *

1 GCCTTCAGATCAGCACTGCCCCCCCCCGAAGTTATTTCTTT--TGCAGGACAGTCAGA 3452
2 GCGAACACCTCAGCACTGCCACCTGTGTACAGTTTTTTCTTGCAATCCCTACAACTGA 4376
3 GACTCCGCCTTTCCACGCCCCCACCTCTGATCTATGATCCTG-----AACTGG 2269
* . : * . . * : * * * * * . . : * : : * * *

1 GCTATATTG-----ATATAGCAAGAG----- 3473
2 GAAAAAAGTTATCTTTTGTAACTTAAACATTCAAATAAAATGTTACAAGTATGTTTTG 4436
3 -ATCTTTAG----- 2277
. : . : : : *

1 -----GTGTGTTACAAAAACACAGGACACTG----- 3500
2 TCCGTGTGTATGTTGGGAGGGCTAATGGATTCTGGGTTTCATGTGGATTTCCTTAGCTTTGC 4496
3 -----CACAAACAGGCCCTCT----- 2294
. . . : . : * * . * *

1 -----TTGAGTTCCTTTGT-----GT 3516
2 GATGACGGGGAAATGGGGTTTGGGTACTTTGGTTAGAAGTGTGGGTTTGGGGTTGGGGA 4556
3 -----TTGAG----- 2299
* * * . *

1 TTGGACTCT-----CCCCTGGG--CG----- 3535
2 TTGAGCTCAGTGGTAGAGCGCTTGCCCTAGGAAGCGCAAGGCCCTGGGTTCCGGTCCCCAGC 4616
3 -----CCTCGTG----- 2306
* * * *

1 -----ACAGTGTTG-----AGGCACTGTTAAG----- 3557
2 TCCGAAAAAAGAACCAAAAAAAAAAAGTGTGGGTTTTCAGCGCAGTTATGCAGAAAAAT 4676
3 -----

1 TCAGGAGCTCAG----- 3569
2 GCAGGTATTCGGTTTGAAGGTAGGACTCATTGGGGGGAACAGTAGGGTGGTGTCTGTAGTT 4736
3 --AAGAATTTGG----- 2316
* . * : . * . *

1 --GGGCCACCG--GTGGATCACTGAAAGCTG--AGACTCTGTTG--CTTCTCCCGTTTG 3620
2 GGGGGTCAGTGGAAAGAGGGACTCTTAGGGCTATGATGACTCAGTTCTCTTAGCCCTCCAG 4796
3 -----ATGTCTG-----TTATTATCATCCACACCCCC 2344
* . * * . * : * * * * : * . . *

1 -----ACACTCTGTTG-----C 3632
2 GGCTGAACCAGAACTGTTTCCCAACTTATGGGTCGAGACCTCTGTTGGAGTCAGACGACC 4856
3 -----CCTT----- 2348
. * *

1 TTTCTTGCATGGTGGCTACCTAAG-----GCTGAGACTCTTG----- 3671

```

2      TTTCCACAGTGGTAGCCTCGGACATTGAAAATTGCAGGGGTTGTTGGTTGTCGGTTGGGT 4916
3      TCACCTCCCTGTGTGGATG-----GTTATTTG----- 2375
      * : ** : : * . *          * : ***

1      -----TTCTCCT-----TCCCTGTATAATCTTGCCTGGCC 3701
2      TTTTGTTTTGTCTTGTTTTGTTCAGACAGGGTCTCTCTGTGTAGCCCTGGCTGGCC 4976
3      -----TCTCTCAGTAAATTTG----- 2391
      * * * : . * . * *

1      -----GTTGCACTTG--TTCCCCAGTGTG----- 3723
2      TCGATTTCGGATCCACATGCCTTTCCCAAACCTGGCTTCCTTATTACTTTTTTTTTTTTT 5036
3      -----TTCCCAA----- 2398
      * * * * . *

1      -----TGAACTCGGAG----- 3734
2      TTTAAGATTTATTTACTTATTATATATAAGTACAATGTAGCTGTCTTCAGAGACACCAGA 5096
3      -----

1      -----ATGAGTTTACACCACCA-----CTG--TTAGT 3759
2      AGAGGGCATCAGATCCCATTACAGATGGCTGTGAGCCACCATGTGGTTGCTGGGATTTGA 5156
3      -----AGG----- 2401
      : *

1      TCACG-----TTTTTTGTTTTTACATAACCATCCTGAAGTCAAGTCAA 3802
2      ACTCAGGACCTCTTCAAGAGCAGTCAGTGCTCTTAAGTCTGAGCCCTCTCTCCAGCCCT 5216
3      AAACG-----TCTGTCACTTTTCGATTTGTTTAG--TCGAGGGCCTT 2441
      : . : *          * : * * * . : : : . : * . . * * :

1      TTTTACTGG-----CTATTTGAAAATAAACTT----- 3830
2      TTATTACTTTTAGAGATTTCTTACCAGGTTGCCCTGGCTGTACTTACACTTTCTCTTTAG 5276
3      ACTGTATATG-----TCTAGCTGACCTGACGCTT----- 2470
      : : * * :          * * . * * : . : : * . * * *

1      -----CAAAGAACTTGCCAG----- 3846
2      CTCTGCTGAGCTTTCAAATTCACCAAGTTCCAGACCACACCCCTGAACCCACCAGCTGAAGA 5336
3      -----ACCGCATTTGGCCAA----- 2484
      . . * . * . * * .

1      -----GTCTTG-----TGTCTGTTGTCTTTTGAGGTCAGGAAT-----TGCTGTGT 3887
2      CAACATGTCTTGCTATCTTTTTCTTTCTTTTGGGGCAGGGATGGTCTGTGTGTTGTGT 5396
3      -----TTTGGTCCTTAACCTTGCAAGCAAT-----CCTCCTGTCT 2517
      * * * * * : * * * . * *          * * * * *

1      ATGT-----CTTACAGA----- 3899
2      GTGTGAGAGACAGAGGCAGAGACATCCTTCCCATGTAGCCCTAACTGAGTCGGAAGTCAT 5456
3      CTG-----CCACCTGAAT----- 2530
      * *          * * * . :

1      --TTGGACTIONGCT-----TGACTIONGATTTTATGGTAAGATGG----- 3936
2      TATTAGACCAGGCTGCCCTGACTCCTGTATCTGCCCTCTCCAATGGAATTATGGGCACCTG 5516
3      --TTCGAGATTACG-----GACCTGCACCATCATG----- 2558
      * * * : . *          . * * . . : * :

1      -----
2      CCACCTCACCCACTCACTTTATCCCCAGGATCCCCCTCCCTCTTCCCAGGCAGTAGGTCT 5576
3      -----

1      ---GGTGGGGAG----- 3945
2      GGTGATGGAGAAGACCACTTACCATGTCCATGGCTCAAAGAAGCAGGCAGAACGTAGGGG 5636
3      -----

1      -----CACCCAGGGACTTTG----- 3960
2      AAAGAGTTGCTTTCACTCAAGCCTTTGGATCCATGGGTGTTTCTTCTAGTGGGGTAATG 5696
3      -----CCCAAC----- 2564
      * * . .

1      -----CATTGGGATCTGCTTG-TGTGAGAAAAATGAAAGCAG-----TCAG 4000

```

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2      CCATCCTTTGAGAGCTGGGGGGTGTGGGTCAACAGAGATCGGTTGCTATGTGTGCATGAG 5756
3      -----TTGTGATTTTTTTTTTTTTTTGGGAGGCAGGGTTTCTTG----- 2600
          ***  **   *           *  ** . * : . . . * . : *

1      ---AGCAAATAACAACCAG---TCCGGG-----GCTGAGGTCTAGT 4035
2      GCCCGCAATTCAGTCCCTGGCTCCATGGAGAACGAACCAATCAGTTGGGTGAGGTGTAAA 5816
3      ---AAGCCTAGGTTAGCCTAG-----AACATTCTAT 2628
          . * * . : :      : :  * * : .          . * . *      : . :

1      ACAGTG-----CAGA----- 4045
2      CCACATGTAATTTTAGCATGTCCAAACAGACACGTTAAGGACATCCTAGGGTAAAGAAAGT 5876
3      ACAC----- 2632
          . **

1      -----
2      C TTCAGCAATAGTTTAATTGAGGCAATTATGCTCATGAATTC 5918
3      -----

```

KEY

- *|| Nucleotides are identical in all sequences in the alignment
- .|| Semi-conserved substitutions are observed
- :|| Conserved substitutions are observed

Appendix 9.17 Amino Acid Sequence for *HSPA1A* (1), *HSPA1B* (2) and *HSPA1L* (3)

Rattus norvegicus

CLUSTAL 2.0.12 multiple sequence alignment

SeqA NameLen(nt) SeqB NameLen(nt) Score

```
=====
1      1      641      2      2      641      100
1      1      641      3      3      641      80
2      2      641      3      3      641      80
=====
```

```

1      --
MAKKTAIGIDLGTTYSCVGVFQHKGVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQ 58
2      --
MAKKTAIGIDLGTTYSCVGVFQHKGVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQ 58
3
MAANKGMAIGIDLGTTYSCVGVFQHKGVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQ 60
      *
*****

1
VALNPQNTVFDAKRLIGRKFGDPVVQSDMKHWPQVVDGDKPKVQVNYKGENRSFYPEE 118
2
VALNPQNTVFDAKRLIGRKFGDPVVQSDMKHWPQVVDGDKPKVQVNYKGENRSFYPEE 118
3
VAMNPQNTVFDAKRLIGRKFNDDPVVQSDMKLWPFQVINEAGKPKVLVSYKGEKKAFYPEE 120
      *:*****.*****
* .*****: :*****

1
ISSMVLTKMKEIAEAYLGHPVTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTA 178
2
ISSMVLTKMKEIAEAYLGHPVTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTA 178
3
ISSMVLTKMKETAFAFLGHSVTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTA 180
      *****
***:***.*****

1
AAIAYGLDRTGKGERNVLIFDLGGGTFDVSILTIDDGIFEVKATAGDTHLGGEDFDNRLV 238
2
AAIAYGLDRTGKGERNVLIFDLGGGTFDVSILTIDDGIFEVKATAGDTHLGGEDFDNRLV 238
3
AAIAYGLDKGSHGERHVLIFDLGGGTFDVSILTIDDGIFEVKATAGDTHLGGEDFDNRLV 240
      *****:
. :***:*****

1
SHFVEEFKRKHKKDISQNKRAVRRLRTACERAKRTLSSSTQASLEIDSLFEGIDFYTSIT 298
2
SHFVEEFKRKHKKDISQNKRAVRRLRTACERAKRTLSSSTQASLEIDSLFEGIDFYTSIT 298
3
SHFVEEFKRKHKKDISQNKRAVRRLRTACERAKRTLSSSTQANLEIDSLYEGIDFYTSIT 300
      *****.*****:*****

1
RARFEELCSDLFRGTLEPVEKALRDAKLDKAQIHDVLVGGSTRIPKVQKLLQDFFNGRD 358
2
RARFEELCSDLFRGTLEPVEKALRDAKLDKAQIHDVLVGGSTRIPKVQKLLQDFFNGRD 358
```

***** • ***** • ***** • ***** • ***** • ***** • *****

2
LNKSI²NPDEAVAYGAAVOAAILMG²DKSEN²VODLL²LLLDVAPLSL²GLETAGGVMTALIK²RNS 418

***** * *****

2
TIP TKOTOTFTTYS DNO PGVLIOVYEGERAMTRDNNLLGRFELSGIPPAPRGVPOIEVTF 478

***** . * . *****

2
DIDANGILNVTATDKSTGKANKITITNDKGRLSKEEIERMVOEAERYKADEVORERVAA 538

* * * . . * *

2
KNALESYAFNMKSAVEDEGLK¹GKISEADKKKVL²DKCOEVISWLD³SN⁴T⁵LAEKEEFVHKREE 598

3 L E N M C N P I I T K L Y Q ~ S G C T G P T C A P G Y T P - G R A A T G P T I E E V D 641
 * * : * * * * * : * * * : * * : * * : : * * * * * * * * *

- *|| Nucleotides are identical in all sequences in the alignment
- .|| Semi-conserved substitutions are observed
- :|| Conserved substitutions are observed

Appendix 9.18 Gene Sequence Alignment for *HSPA1A* *Homo sapiens* v *Rattus norvegicus*

CLUSTAL 2.0.12 Multiple Sequence Alignments

```
SeqA NameLen(nt)  SeqB NameLen(nt)  Score
=====
1      1      2445      2      2      4045      73
=====
```

```
1      -----
2      CCAGGCATCCTTCTCTGAGTTTCTATCTTTCTCTCGATTGTAACTCCTCTCTCGACCAT
60
```

```
1      -----
2      CAATACCATAATCAAAAAGGTCTTGTCTGCCTCCGATTTAGCTCAGACTAAATTCAGCC
120
```

```
1      -----
2      ACTAATGTCCCTCAAGAGCCCAACCCATTTTCTTGGTTGCCAACACCCAAATCCAGA
180
```

```
1      -----
2      ATTAAACTGGGGTTCCTGTGTGGAGAGCCAAGAGGAGACCTAAGGATGGTGGCTTCCAC
240
```

```
1      -----
2      TAGACCACGTCTGTAATCAATCAAACCTAAGAAAATTCTTGGCCCATTAATAAGAACC
300
```

```
1      -----
2      AACTGGAATTCCAGGCTCACCTGGAATCTCTACGCCTTCGATCCAGTTTGGAAAAATTTGA
360
```

```
1      -----
2      AGTCGCTGAGCCCCCTACGAGTGGGGAGCTCCAGGAACATCCAAACTGAGCAACTGGGGTT
420
```

```
1      -----
2      CCCCCACCCCCACCCGCCCCCTCCGGCAACTTTGAGCCTCTTCTGGGACAGATCCTC
480
```

```
1      -----
2      TGATTCTTAATAAGTCCATGAGGTCAGAGTTAGCACTGCCATTGTAACGCGCTGGAGG
540
```

```
1      -----
2      GGGGTCACGTCACCGGACACGCCCCAGGCACCTCCCTTGGGTCTCCCAAGGCTAGGAAG
600
```

```
1      -----
2      GGAAGTTATAACCCCTTAACCTCGAGCCCCATAATCAGAACTGTGCGAGTCTGCGAACCC
660
```



```

1 -----
2 CACAAATCACAACCAACTGTCCACAACACGGAGCTAGCAGTGACCTTTCCTGTCCATTCC
720

1 -----
2 ACTCAGGCCTCAGTAATGCGTCGCCATAGCAACAGTGTCAACAGCAGCACCAGCAGGTCC
780

1 -----
2 CGCCACCCTCCCCACAGGAATCCGTCCTTCCAGCTAACCCAGATCTGTCTGGAGAGT
840

1 -----
2 TCTGAACAGGGGCGGAACCTCAACTCCTATTACTCAAAGGAGGCGGGGAAGCTCCACCC
900

1 -----
2 GACGCGAAACTGCTGGAAGATTCCCTGGCCCCAAGGCCTCCTCCGCTCGCTGATTGGCCC
960
                                     *      * * * *

1 AGGGGCAAGCGGTCCGGAT---AACGGCTAGCCTGAGGAGCTG--CTGCG-ACAGTCCA
63
2 ATGGGAGGGTGGGCGGGGCCGGAGGAGGCTCCTTAAAGGCGCAGGGCGGGCGCAGGACA
1020
* * * *      * * * *      * * * *      * * * *      * * * *      * *

1 CTA---CCTTTTTCGAGAGTGACTCCCGTTGTCCCAGGCT--TCCCAGAGC-----
110
2 CCAGATTCTCTCTTTAATCTGACAGAATCAGTTTCTGGTTCCACTCGCAGAGAAGCAGA
1080
* *      * * * *      * * * *      * * * *      * * * *      * * * *

1 GAACCTGTGCG-GCTGCAGGCACCGGCGCGTTCGAGT-----TTCCGGCGT---
154
2 GAAGCGGAGCAAGCGGCGCGTTCCAGAACCTCGGGCAAGACCAGCCTCTCCCAGAGCATC
1140
* * * *      * * * *      * * * *      * * * *      * * * *      * * * *

1 ----CCGGAAGGACCGAGCTCTTCTCGCGGATCC-----AGTGTTCGTTTCC--A
199
2 CCCACCGCGAAGCGCAACCTTCTCCAGAGCATCCCCAGCGGAGCGCACCCCTTCCCCAGA
1200
* * * *      * * * *      * * * *      * * * *      * * * *      * * * *

1 GC-CCCCAATCTCAGAGCGGAGCCGACAGAGAGCAGGGAAC--CGGCATGGCCAAAGCCG
256
2 GCATCCCCGCGGCCAAGCGCAACCTTCCAGAAGCAGAGAGCGGCGACATGGCCAAAGAAAA
1260
* *      * * * *      * * * *      * * * *      * * * *      * * * *

1 CGGCGATCGGCATCGACCTGGGCACCACTACTCCTGCGTGGGGGTGTTCCAACACGGCA
316
2 CAGCGATCGGCATCGACCTGGGCACCACTACTCCTGCGTGGGGGTGTTCCAACACGGCA
1320
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

1 AGGTGGAGATCATCGCCAACGACCAAGGGCAACCGCACCAACCCCAAGCTACGTGGCCTTCA
376
2 AGGTGGAGATCATCGCCAACGACCAAGGGCAACCGCACCAACCCCAAGCTACGTGGCCTTCA
1380
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

1 CGGACACCGAGCGGCTCATCGGGGATGCGGCCAAGAACCAGGTGGCGCTGAACCCGCGAGA
436

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[illegible]

1 ACTCCCTGTTTGAGGGCATCGACTTCTACACGTCCATCACAGGGGCGAGGTTCGAGGAGC
1156
2 ACTCTCTGTTTCGAGGGCATCGACTTCTACACGTCCATCACGCGGGCGCGGTTTCGAGGAGC
2160

1 TGTGCTCCGACCTGTTCCGAAGCACCCCTGGAGCCCGTGGAGAAGGCTCTGCGCGACGCCA
1216
2 TGTGCTCGGACCTGTTCCGCGGCACGCTGGAGCCCGTGGAGAAGGCCCTGCGCGACGCCA
2220

1 AGCTGGACAAGGCCAGATTACAGACCTGGTCCTGGTCGGGGGCTCCACCCGCATCCCCA
1276
2 AGCTGGACAAGGCGCAGATCCACGACCTGGTGCTGGTGGGCGGCTCGACGCGCATCCCCA
2280

1 AGGTGCAGAAGCTGCTGCAGGACTTCTTCAACGGGCGCGACCTGAACAAGAGCATCAACC
1336
2 AGGTGCAGAAGCTGCTGCAGGACTTCTTCAACGGGCGCGACCTGAACAAGAGCATCAATC
2340

1 CCGACGAGGCTGTGGCCTACGGGGCGGCGGTGCAGGCGGCCATCCTGATGGGGGACAAGT
1396
2 CCGACGAGGCGGTGGCCTACGGGGCGGCGGTGCAGGCGGCCATCCTGATGGGGGACAAGT
2400
* *****

1 CCGAGAACGTGCAGGACCTGCTGCTGCTGGACGTGGCTCCCCTGTCGCTGGGGCTGGAGA
1456
2 CCGAGAACGTGCAGGACCTGCTGCTGCTGGACGTGGCGCCGCTGTCGCTGGGTCTGGAGA
2460
* *****

1 CGGCCGGAGGCGTGATGACTGCCCTGATCAAGCGCAACTCCACCATCCCCACCAAGCAGA
1516
2 CGCGGGCGGCGTGATGACGCGCTCATCAAGCGCAACTCCACCATCCCCACCAAGCAGA
2520
* * * *****

1 CGCAGATCTTCAACACCTACTCCGACAACCAACCCGGGGTGCTGATCCAGGTGTACGAGG
1576
2 CGCAGACCTTCAACACCTACTCGGACAACCAAGCCGGGGTGCTGATCCAGGTGTACGAGG
2580

1 GCGAGAGGGCCATGACGAAAGACAACAATCTGTTGGGGCGCTTCGAGCTGAGCGGCATCC
1636
2 GCGAGAGGGCCATGACGCGCGACAACAACCTGCTGGGGCGCTTCGAGTTGAGCGGCATCC
2640

1 CTCGGCCCCCAGGGGCGTGCCCCAGATCGAGGTGACCTTCGACATCGATGCCAACGGCA
1696
2 CGCCGGCTCCCAGGGGCGTGCCCCAGATCGAGGTGACCTTCGACATCGACGCCAACGGCA
2700
* *****

1 TCCTGAACGTCACGGCCACGGAACAGCACCGGCAAGGCCAACAGATCACCATCACCA
1756
2 TCCTGAACGTCACGGCCACTGACAAGAGCACCGGCAAGGCCAACAGATCACCATCACCA
2760

1 ACGACAAGGGCCGCCTGAGCAAGGAGGAGATCGAGCGCATGGTGCAGGAGGCGGAGAAGT
1816
2 ACGACAAGGGCCGCCTGAGCAAGGAGGAGATCGAGCGCATGGTGCAGGAGGCCGAGCGCT
2820

```

*****
1      ACAAAGCGGAGGACGAGGTGCAGCGCAGAGGGTGTCAGCCAAGAACGCCCTGGAGTCCT
1876
2      ACAAGGCGGAGGACGAGGTGCAGCGCAGAGGGTGGCTGCCAAGAATGCGCTCGAGTCCT
2880
****
1      ACGCCTTCAACATGAAGAGCGCCGTGGAGGATGAGGGGCTCAAGGGCAAGATCAGCGAGG
1936
2      ATGCCTTCAACATGAAGAGCGCCGTGGAGGACGAGGGTCTCAAGGGCAAGATCAGCGAGG
2940
*
1      CGGACAAGAAGAAGGTGCTGGACAAGTGTC AAGAGGTCACTCTCGTGGCTGGACGCCAACA
1996
2      CTGACAAGAAGAAGGTGCTGGACAAGTGCCAGGAGGTCACTCTCTGGCTGGACTCTAACA
3000
*
1      CCTTGGCCGAGAAGGACGAGTTTGAGCACAAAGAGGAAGGAGCTGGAGCAGGTGTGTAACC
2056
2      CGCTGGCTGAGAAAGAGGAGTTTCGTGCACAAGCGGGAGGAGCTGGAGCGGGTGTGCAACC
3060
*
1      CCATCATCAGCGGACTGTACCAAGGTGCCGGTGGTCCCGGGCCTGGGGGCTTCGGGGCTC
2116
2      CGATCATCAGCGGGCTGTATCAGGGTGCGGGTGCTCCCGGGCTGGGGGCTTCGGGGCCC
3120
*
1      AGGGTCCC AAGGAGGGTCTGGGTCAAGCCCCACCATGAGGAGGTAGATTAGGGGC-CT
2175
2      AGGCGCCC AAGGAGGCCTCTGGGTGCGGGCCCACCATCAGGAGGTGGATTAGAGGCTCT
3180
***
1      TTCCAAGATTGCTGTTTTTTGTTTGGAG-----CTTCAAGACTTTGCATTTTC
2222
2      TTCTGGCGCTCCAGGTGTGATCTAGGAGACAGATGGGTGGCCTTGAGGACTTTGGGTTAT
3240
***
1      C-----TAGTATTTCTGTTTGTCAAGTCTCAATT---TCCTGTGTTT--GCAA-
2265
2      TGTCGTTTAGGACATTAACTCCTTCGTTCGGTCTGCAATCAAGTCCTAGGTTTAAGCAAA
3300
*
1      ---TGTGAAATTTTTTGGTGAAGTACTGAACTTGCTTTT---TTTCCGGTTTCTACATGC
2320
2      CTGCCTTCCATTTACTCTGTGGAATTTACGCTGTGCTTTGCATTCCCAGTAAATTAGTAC
3360
*
1      AGAGA-TGAATTTATACTGCCATCTTACGACTATTTCTCTTTTTAATACACTTAATC-
2378
2      TGGGAGTGTGCTTTGCAATAGATATAATTTCCTGCCTTCAAGTCAGCACTGCCCCCCCC
3420
*
1      ---AGGCCATTTTTTAAGTTGGTTACTTCAAAGTAAATAAACTT--TAAATTCAAAAA
2432
2      CCCGAAGTTATTTCTTTTGCAGGACAGTCAGAGCTATATTGATATAGCAAGAGGTGTGTT
3480
*
1      AAAAAAAAAA-----
2445

```

```

2      AAAAAACACCAAGGACACTGTTGAGTTCCTTTGTGTTTGGACTCTCCCCTGGGCGACAGT
3540
      * * * * * * *
1
-----
2      GTTGAGGCAC TGTTAAGTCAGGAGCTCAGGGGCCACCGGTGGATCACTGAAAGCTGAGAC
3600

1
-----
2      TCTGTTGCTTCTCCGTTTGACACTCTGTTGCTTTCCTTGCA TGGTGGCTCACCTAAGGC
3660

1
-----
2      TGAGACTCTTGTTCTCCTTCCTGTATAATCTTGCCTGGCCGTTGCAC TTGTTCCCCAGT
3720

1
-----
2      GTGTGAACTCGGAGATGAGTTTACACCACCACTGTTAGTTCACGTTTTTTGTTTTTACAT
3780

1
-----
2      AACCATCCTGA ACTCAGGTCAATTTTTACTGGCTATTTGAAATAAACTTCAAAAGAACT
3840

1
-----
2      TGCCAGGTCTTG TGTCTGTTGTCTTTTGAGGTCAGGAATTGCTGTGTATGTCTTACAGAT
3900

1
-----
2      TGGACTACGCTTGACTGACATTTTATGGTAAGATGGGGTGGGGAGCACCCAGGGACTTTG
3960

1
-----
2      CATTGGGATCTGCTTGTGTGAGAAAAATGAAAGCAGTCAGAGCAATAACAACCAAGTCCG
4020

1
-----
2      GGGCTGAGGTCTAGTACAGTGCAGA 4045

```

KEY

- *|| Nucleotides are identical in all sequences in the alignment
- .|| Semi-conserved substitutions are observed
- :|| Conserved substitutions are observed

Appendix 9.19 Amino Acid Sequence for HSPA1A Homo sapiens v Rattus norvegicus

CLUSTAL 2.0.12 multiple sequence alignment

```

SeqA NameLen(aa)  SeqB NameLen(aa)  Score
=====
1      1    641      2      2    641      96
=====

1      MAKAAAI GIDL GTTYSCVGVFQH GKVEII ANDQGN R TTPSYVAF T DTERLIGDA AKNQVA
60
2      MAKKTAIGIDL GTTYSCVGVFQH GKVEII ANDQGN R TTPSYVAF T DTERLIGDA AKNQVA
60
      *** : *****

1      LNPQNTVFD AKRLIGRKFGDPV VQSDMKHWP FQVINDGDKPKVQVSYKGETKAFY PEEIS
120
2      LNPQNTVFD AKRLIGRKFGDPV VQSDMKHWP FQVNDGDKPKVQVNYKGENRSFY PEEIS
120
      ***** : *****

1      SMVLTKMKEIAEAYLGYPVTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTAAA
180
2      SMVLTKMKEIAEAYLGHPVTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTAAA
180
      ***** : *****

1      IAYGLDRTGKGERNVLIFDLGGGTFDVSILTIDDGIFEVKATAGDTHLGGEDFDNRLNVH
240
2      IAYGLDRTGKGERNVLIFDLGGGTFDVSILTIDDGIFEVKATAGDTHLGGEDFDNRLVSH
240
      *****

1      FVEEFKRKHKKDISQNKRAVRRLRTACERAKRTLSSSTQASLEIDSLFEGIDFYTSITRA
300
2      FVEEFKRKHKKDISQNKRAVRRLRTACERAKRTLSSSTQASLEIDSLFEGIDFYTSITRA
300
      *****

1      RFEELCSDLFRSTLEPVEKALRDAKLDKAQIHD LVLVGGSTRIPKVQKLLQDFFNGRDLN
360
2      RFEELCSDLFRGTLEPVEKALRDAKLDKAQIHD LVLVGGSTRIPKVQKLLQDFFNGRDLN
360
      *****

1      KSINPDEAVAYGA AVQAAILMGDKSENVQD LLLL DVAPLSLGLETAGGVM TALIKRNSTI
420
2      KSINPDEAVAYGA AVQAAILMGDKSENVQD LLLL DVAPLSLGLETAGGVM TALIKRNSTI
420
      *****

1      PTKQTQIFTTYS DNQPGVLIQVYEGERAMTKDNNLLGRFELSGIPPAPRGVPQIEVTFDI
480
2      PTKQTQTFTTYS DNQPGVLIQVYEGERAMTRDNNLLGRFELSGIPPAPRGVPQIEVTFDI
480
      *****

1      DANGILNVTATDKSTGKANKITITNDKGRLSKEEIERMVQEA EKYKAEDVQRERVSAKN
540
2      DANGILNVTATDKSTGKANKITITNDKGRLSKEEIERMVQEA ERYKAEDVQRERVAAKN
540
      *****

1      ALESYAFNMKSAVEDEGLKGKISEADKKKVLDKCQEVISWLDANTLAEKDEF EHKRKELE
600
2      ALESYAFNMKSAVEDEGLKGKISEADKKKVLDKCQEVISWLD SNTLAEKEEFVHKREELE
600

```

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*****:*****:***:***
1      QVCNPIISGLYQGAGGPGGGFGA 624
2      RVCNPIISGLYQGAGAPGAGGFGA 624
      :*****_*_*_*

```

KEY

- *|| Nucleotides are identical in all sequences in the alignment
- .|| Semi-conserved substitutions are observed
- :|| Conserved substitutions are observed

| | |
|----------|-----------------------------|
| MVFILAPW | Non polar R-groups |
| GNTSYQHC | Polar R-groups |
| KR | Positively charged R-groups |
| DE | Negatively charged R-groups |

Appendix 9.20 Gene Sequence Alignment for *HSPA1B* *Homo sapiens* v *Rattus norvegicus*

| SeqA | NameLen(nt) | SeqB | NameLen(nt) | Score |
|-------|-------------|--|-------------|-------|
| ===== | | | | |
| 1 | 1 | 2 | 2 | 5918 |
| 2551 | | 5918 | | 78 |
| ===== | | | | |
| 1 | | ----- | | |
| 2 | | GAATTCCAGCACTTGGGTGTCAAGGCTGGAGGAGCAAGAGTTTAAGGCTAGCCTGGGCTA | | |
| 60 | | | | |
| 1 | | ----- | | |
| 2 | | CATGAGCCCTATTTTGAAAAAGAAAACAAGAAATAAAAAAGTTAGAAAAAGAAAAAAT | | |
| 120 | | | | |
| 1 | | ----- | | |
| 2 | | GAAACAAAAGATGTGTGCGTGTGTATGTGTGTGAGAGAGAGACAGTCTCTCTCTCTC | | |
| 180 | | | | |
| 1 | | ----- | | |
| 2 | | TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTGTGTATGTGTGTGTGTGAG | | |
| 240 | | | | |
| 1 | | ----- | | |
| 2 | | ACAATTACTCCCTGCTGTGGAAGTGTACCTCAGAACTAAGACTCTAATGCATCTATTCT | | |
| 300 | | | | |
| 1 | | ----- | | |
| 2 | | TTTTTTAAAAAACAGGCTCGGGGCTGGGGATTTAGCTCAGTGGTAGAGCGCTTACCTAGG | | |
| 360 | | | | |
| 1 | | ----- | | |
| 2 | | AAGCGCAAGGCCCTGGGTTCGGTCCCCAGCTCCGAAAAAAGAACCAAAAAAAAAAAAAA | | |
| 420 | | | | |
| 1 | | ----- | | |
| 2 | | ACCAAAACAAAAAAAAAAAAACAAAAAAAAACAAACAAACAGGCTCTAATGTAGATTG | | |
| 480 | | | | |
| 1 | | ----- | | |
| 2 | | GGCTAGCCTCAAACTCTTCATCCTCCTGCCTCCCCCTCCAAAGCATGAGGACTACAGAGG | | |
| 540 | | | | |
| 1 | | ----- | | |
| 2 | | TGGGCCACCATACCCAGTAACCTCATACTGTTTCAAGACATCTAACTAGGGGGTCTTTAA | | |
| 600 | | | | |
| 1 | | ----- | | |
| 2 | | TTCGTACACGGAAGGAGGAGCCCGGAAGAAAGAAAAGGGACATCACTGACAGCTAAACAT | | |
| 660 | | | | |
| 1 | | ----- | | |
| 2 | | ATTTATAATGAGAGGAGGTAGGGGACAGACAAGGGACGGAAGGTGATGTCTAGGACAAA | | |
| 720 | | | | |

1
2
780

AAGCTAACCA~~CATAA~~ACTAGTAGGTGGGTCCCGA~~CAC~~CTCTGCTTAGGGTCTCA~~CCCAA~~

1
2
840

AGTCGGAGTTAACAGCACTGTGAGGCCAGATGCTATCAGAGGACAGGACAC~~CCATCTCT~~

1
2
900

CCACCTAGGAAAGGTGAACTGACAGGGGCAGCAGACACAGAAGAAATCAGCAAACTCTTAA

1
2
960

GTCAACCCTGAGGTTACTACAGCACAGAATAGAACAGTGCC~~TGGCAAGGAGCAGCTGTGT~~

1
2
1020

GAAGGAGAA~~GCCAAGCAGCCAT~~CCTCAGCATTCCTTCTTCTATTAAATCTTTTGGGACAGGG

1
2
1080

TCTGCCTCTGTAA~~CCTAGGCTGACCTTGAC~~TGCTGCAGTCCCTCTGCCCCA~~CCCTCTAC~~

1
2
1140

CGAAAGTTTTTTTTTTCTCCTTTCTAATGCCAGGCTGCCGCTGAGGCA~~AAAGGGAGCGG~~

1
2
1200

CTTGCA~~GCCTGGCACGGTCGGTCGATTAA~~ACCTTGCTCTCCCCGCTCTGGGAC~~ACTTTCC~~

1
2
1260

TTTTTTGGGTCACAGGTCCTCCTAA~~CATGAGA~~ACCGAGTGTTACACAATGATGTTCTTT

1
2
1320

TGAAAACCGTGAAA~~ACTCCACAGGCGATGTACT~~TGTACGTTAAGCGTGACATAAAGACAG

1
2
1380

CAAAGCGAATAAACTATACTGCAGATCTCTCTCTTTCCCTATTTAAACCTAAAATGGA

1
2
1440

GGGAGTGGGGGGCAGACACAGACAGGCGAGCATTCACAGGCGCCCCCCCCACGCTGTCA

1
2
1500

CTTCCAGGCAGGACCCAA~~TACAGACTTCTTAGCC~~AAGCGTTATCCCTCCCGTTTTGAGA

1
2
1560

AACTTTCTGCGTCCGCCATCCTGTAGGAAGAA~~TTTGTA~~CACCTTAAACTCCCTCCCTGGT

```

1 -----
2 CTGATTCCCAAATGTCTCTCACCGCCCAGCACTTTCAGGAGCTGACCCTTCTCAGCTTCA
1620

1 -----
2 CATACAGAGACCGCTACCTTGCCTCGCCATGGCAACACTGTACAACCGAACAAGCACT
1680

1 -----
2 TCCTACCACCCCCCGCCTCAGGAATCCAATCTGTCCAGCGAAGCCCAGATCCGTCTGGAG
1740

1 -----
2 AGTTCTGGACAAGGGCGGTACCTCAACATGGATTACTCATGGGAGGCGGAGAAGCTCTA
1800

1 -----
2 ACAGACCCGAAACTGCTGGAAGATTCTGGCCCCAAGGCCTCTCCGCTCGCTGATTGG
1860

1 -----
31 -----GGAAAACGGCC-----AGCCTGAGGAGCTGCTGCGA
2 CCCATGGGAGGGTGGGCGGGCGGAGGAGGCTCCTTAAAGGCGCAGGGCGGCGCGCAGG
1920
                * * * * * * * * * *

1 GGGTCCGCTTCGTCTTTCGAGAGTACTCCCGCGGTCCCAAGGCTTTCCAG-AGCGAACC
90
2 ACACCAGATTCTCTCCTCTAACTGACAGAACCAGTTTCTGGTTCCACTCGCAGAGAAGC
1980
                * * * * * * * * * * * * * * * * * * * * * *

1 TGTGCGGCTGCAGGCACCGGCGTGTT--GAGTTTCCGGCG-----TTCCGAAG
136
2 AGAGAAGC-GGAGCAAGCGGCGCGTTCCAGAACCTCGGGCAAGACCAGCCTCTCCCAGAG
2039
                * * * * * * * * * * * * * * * * * * * *

1 GACT-----GAGCTCTTGTCGCGGATCCC-----GTCCGCCGTTTCC
173
2 CATCCCCACCGCGAAGCGCAACCTTCTCCAGAGCATCCCCAGCGGAGCGCACCTTCCC
2099
                * * * * * * * * * * * * * * * * * * * *

1 ---AGC-CCCCAGTCTCAGAGCGGAGCCCAC---AGAGCAGGGCACCGGCATGGCCAAA
225
2 CAGAGCATCCCCCGCGCCAAAGCGCAACCTTCCAGAAGCAGAGAGCGGCGACATGGCCAAG
2159
                * * * * * * * * * * * * * * * * * * * *

1 GCCGCGGCGATCGGCATCGACCTGGGCACCACTACTCCTGCGTGGGGTGTTCCAACAC
285
2 AAAACAGCGATCGGCATCGACCTGGGCACCACTACTCGTGCCTGGGCGTGTTCAGCAC
2219
                * * * * * * * * * * * * * * * * * * * *

1 GGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGCACCACCCCAGCTACGTGGCC
345
2 GGCAAGGTGGAGATCATCGCCAACGACCAGGGCAACCGCACCACCCCAGCTACGTGGCC
2279
                * * * * * * * * * * * * * * * * * * * *

```

1 TTCACGGACACCGAGCGGCTCATCGGGGATGCGGCCAAGAACCAAGGTGGCGCTGAACCCG
405
2 TTCACCGACACCGAGCGGCTCATCGGGGACGCCGCCAAGAACCAAGGTGGCGCTGAACCCG
2339

1 CAGAACACCGTGTTTGACGCGAAGCGGCTGATCGGCCGCAAGTTCGGCGACCCGGTGGTG
465
2 CAGAACACCGTGTTCGACGCGAAGCGGCTGATCGGCCGCAAGTTCGGCGACCCGGTGGTG
2399

1 CAGTCGGACATGAAGCACTGGCCTTTCCAGGTGATCAACGACGGAGACAAGCCCAAGGTG
525
2 CAGTCGGACATGAAGCACTGGCCCTTCCAGGTGGTGAACGACGGCGACAAGCCCAAGGTG
2459

1 CAGGTGAGCTACAAGGGGGAGACCAAGGCATTCTACCCCGAGGAGATCTCGTCCATGGTG
585
2 CAGGTGAACTACAAGGGCGAGAACCGGTCGTTCTACCCCGAGGAGATCTCGTCCATGGTG
2519

1 CTGACCAAGATGAAGGAGATCGCCGAGGCGTACCTGGGCTACCCGGTGACCAACGCGGTG
645
2 CTGACCAAGATGAAGGAGATCGCCGAGGCGTACCTGGGCCACCCGGTGACCAACGCGGTG
2579

1 ATCACCGTGCCGGCCTACTTCAACGACTCGCAGCGCCAGGCCACCAAGGATCGGGGTGTG
705
2 ATCACCGTGCCGCCTACTTCAACGACTCGCAGCGGCAGGCCACCAAGGACGCGGGCGTG
2639

1 ATCGCGGGGCTCAACGTGCTGCGGATCATCAACGAGCCCACGGCCGCCATCGCCTAC
765
2 ATCGCGGGTCTGAACGTGCTGCGGATCATCAACGAGCCCACGGCGGCCGCCATCGCCTAC
2699

1 GGCCTGGACAGAACGGGCAGGGGGAGCGCAACGTGCTCATCTTTGACCTGGGCGGGGGC
825
2 GGGCTGGACCGGACCGGCAGGGCGAGCGCAACGTGCTCATCTTCGACCTGGGGGGCGGC
2759
**

1 ACCTTCGACGTGTCCATCCTGACGATCGACGACGGCATCTTCGAGGTGAAGGCCACGGCC
885
2 ACGTTCGACGTGTCCATCCTGACGATCGACGACGGCATCTTCGAGGTGAAGGCCACGGCG
2819
**

1 GGGGACACCCACCTGGGTGGGGAGGACTTTGACAACAGGCTGGTGAACTTCGTGGAG
945
2 GCGGACACGCACTGGGCGGGGAGGACTTCGACAACCGGCTGGTGAGCCACTTCGTGGAG
2879
**

1 GAGTTCAAGAGAAAACACAAGAAGGACATCAGCCAGAACAAGCGAGCCGTGAGGCGGCTG
1005
2 GAGTTCAAGAGGAAGCACAGAAGGACATCAGCCAGAACAAGCGCGCGGTGCGGCGACTG
2939

1 CGCACCGCCTGCGAGAGGGCCAAGAGGACCCTGTCTGTCCAGCACCCAGGCCAGCCTGGAG
1065
2 CGCACGGCGTGCGAGAGGGCCAAGAGGACGCTGTCTGTCCAGCACCCAGGCCAGCCTGGAG
2999

```

*****  ** *****
1      ATCGACTCCCTGTTTGAGGGCATCGACTTCTACACGTCCATCACAGGGCGAGGTTTCGAG
1125
2      ATCGACTCTCTGTTTCGAGGGCATCGACTTCTACACGTCCATCACGCGGGCGCGGTTTCGAG
3059
*****  ***** *****
1      GAGCTGTGCTCCGACCTGTTCGAGACCCCTGGAGCCCGTGGAGAAGGCTCTGCGCGAC
1185
2      GAGCTGTGCTCGGACCTGTTCGCGGCACGCTGGAGCCCGTGGAGAAGGCCCTGCGCGAC
3119
*****  ***** *****
1      GCCAAGCTGGACAAGGCCAGATTACGACCTGGTCCTGGTCGGGGGCTCCACCCGCATC
1245
2      GCCAAGCTGGACAAGGCGCAGATCCACGACCTGGTGCTGGTGGGCGGCTCGACGCGCATC
3179
*****  ***** ***** ** ***** ** *****
1      CCCAAGGTGCAGAAGCTGCTGCAGGACTTCTTCAACGGGCGCGACCTGAACAAGAGCATC
1305
2      CCCAAGGTGCAGAAGCTGCTGCAGGACTTCTTCAACGGGCGCGACCTGAACAAGAGCATC
3239
*****
1      AACCCCGACGAGGCTGTGGCCTACGGGGCGGCGGTGCAGGCGGCCATCCTGATGGGGGAC
1365
2      AATCCGGACGAGGCGGTGGCCTACGGGGCGGCGGTGCAGGCGGCCATCCTGATGGGGGAC
3299
** ** *****
1      AAGTCCGAGAACGTGCAGGACCTGCTGCTGCTGGACGTGGCTCCCCTGTCGCTGGGGCTG
1425
2      AAGTCGGAGAACGTGCAGGACCTGCTGCTGCTGGACGTGGCGCCGCTGTCGCTGGGTCTG
3359
***** ***** ** *****
1      GAGACGCGCCGAGGCGTGATGACTGCCCTGATCAAGCGCAACTCCACCATCCCCACCAAG
1485
2      GAGACGCGGGCGGCGTGATGACGGCGCTCATCAAGCGCAACTCCACCATCCCCACCAAG
3419
***** ** ** ***** ** *****
1      CAGACGCAGATCTTACCACCTACTCCGACAACCAACCCGGGGTGCTGATCCAGGTGTAC
1545
2      CAGACGCAGACCTTACCACCTACTCGGACAACAGCCCGGGTGCTGATCCAGGTGTAC
3479
***** ***** *****
1      GAGGGCGAGAGGGCCATGACGAAAGACAACAATCTGTTGGGGCGCTTCGAGCTGAGCGGC
1605
2      GAGGGCGAGAGGGCCATGACGCGGACAACAACCTGCTGGGGCGCTTCGAGTTGAGCGGC
3539
***** *****
1      ATCCCTCCGGCCCCAGGGGCGTGCCCCAGATCGAGGTGACCTTCGACATCGATGCCAAC
1665
2      ATCCCGCCGGCTCCCAGGGGCGTGCCCCAGATCGAGGTGACCTTCGACATCGACGCCAAC
3599
***** *****
1      GGCATCCTGAACGTACGGCCACGGACAAGAGCACCGGCAGGGCCAACAAGATCACCATC
1725
2      GGCATCCTGAACGTACGGCCACTGACAAGAGCACCGGCAGGGCCAACAAGATCACCATC
3659
*****
1      ACCAACGACAAGGGCCGCCTGAGCAAGGAGGAGATCGAGCGCATGGTGCAGGAGGCGGAG
1785

```

2
3719
ACCAACGACAAGGGCCGCCTGAGCAAGGAGGAGATCGAGCGCATGGTGCAGGAGGCCGAG

1
1845
AAGTACAAAGCGGAGGACGAGGTGCAGCGCAGAGGGTGTCAAGCAAGAACGCCCTGGAG
2
3779
CGCTACAAGGCGGAGGACGAGGTGCAGCGCAGAGGGTGGCTGCCAAGAATGCGCTCGAG

1
1905
TCCTACGCCTTCAACATGAAGAGCGCCGTGGAGGATGAGGGGCTCAAGGGCAAGATCAGC
2
3839
TCCTATGCCTTCAACATGAAGAGCGCCGTGGAGGACGAGGGTCTCAAGGGCAAGATCAGC

1
1965
GAGGCGGACAAGAAGAAGGTCTGGACAAGTGTCAGAGGTCACTCGTGGCTGGACGCC
2
3899
GAGGCTGACAAGAAGAAGGTGCTGGACAAGTGCCAGGAGGTCACTCCTGGCTGGACTCT

1
2025
AACACCTTGCCGAGAAGGACGAGTTTGAGCACAAGAGGAAGGAGCTGGAGCAGGTGTGT
2
3959
AACACGCTGGCTGAGAAAGAGGAGTTCTGTGCACAAGCGGGAGGAGCTGGAGCGGGTGTGC

1
2085
AACCCCATCATCAGCGGACTGTACCAGGGTGCCGGTGGTCCCGGGCCTGGCGGCTTCGGG
2
4019
AACCCGATCATCAGCGGGCTGTATCAGGGTGCGGGTGCTCCCGGGGCTGGGGGCTTCGGG

1
2145
GCTCAGGGTCCCAGGGAGGGTCTGGGTACAGCCCTACCATAGGAGGTGGATTAGGGG
2
4079
GCCCAGGCGCCCAGGGAGGCTCTGGGTGCGGGGCCACCATCGAGGAGGTGGATTAGAGG
** *****

1
2201
CCTTTGT--TCTTTAGTATGTTTGTCTTTGAGGTGGACTGTG--GGACTCAAGGACTTT
2
4137
CTTTTCTGGCTCTCAGGGTGTGG--CTAGAGACAGACTCTTGATGGCTGCTGGTGCACG
* * * * *

1
2259
GCTGCTGTTTCTCTATGTCAATTTCTGCTTCAGCTC--TTTGCTGCTTCACTTCTTTGTAA
2
4196
ATTCTTATCAAGTTACTCCTTCTCT--CCGGAGTTCAAGTTTAAAGTTACAGCCTTTTATAC
* * *

1
2314
AGTTGT-AACCTGA--TGGTA-ATTAGCTGGCTTCATTATTTTTGTAGTACAACCGATA
2
4255
GGTAATTGATTGAGTTTGTACATTTTGTATGCTCGTGGGTTTTTTA-TATATTCAAAT
* * *

1
2368
TGTTCAATTAGAATTCTTTGCATTTAATGTTGATA-CTGT----AAGGGTGTTCGTTCC
2
4315
TAAGGTTGCATGTTCTTTGCGTTTAATCTAAGTAGCTGTGTAAAAATGGTGTTCCTTCC
* * *

1
2428
CTTTAAATGAATCAACACTGCCACCTTCTGTACGAGTTTGTGTGTTTTTTTTTTTTTTTT
2
4373
-TGCGAACACCTCAGCACTGCCACCCTGTGTAC-AGTTTTTTCCTTGCATCCCTACAAAC
* * *

```

1      T-----T-----TTTTTTTGCTTGGCGAAAACTACAAA--GGCTGGGA-ATGTATGTT
2474
2      TGAGAAAAAAGTTATCTTTTGTAACTTAAACATTCAAATAAAATGTTACAAGTATGTT
4433
      *               ** * * *      *      *      * * * * *      * * *      * * * * * * * *
1      TTTATAATTGTTTATTTAAAT-----ATGAAAAATAAA--ATGTTAACTTTAAAAAA
2526
2      TTGTCCGTGTGTATGTTGGGAGGGCTAATGGAATTCTGGGTTCAATGTTGATTTCTTAGCTT
4493
      **           * * * * *      *      * * *      *      * * *      * * * *
1      AAAAAAAAAAAAAAAAAAAAAA-----
2551
2      TCGCATGACGGGGAATGGGGTTTGGGTACTTTGGTTAGAACTGTGGGTTTGGGGTTGG
4553
      *      *      * * *
1      -----
2      GGATTGAGCTCAGTGGTAGAGCGCTTGCCTAGGAAGCGCAAGGCCCTGGGTTCGGTCCCC
4613
1      -----
2      AGCTCCGAAAAAGAACCAAAAAAAAAAAAAGTGTGGGTTTTCAGCGCAGTTATGCAGAA
4673
1      -----
2      AATGCAGGTATTCGGTTTGAGGGTAGGACTCATTGGGGGAACAGTAGGGTGGTGTGTA
4733
1      -----
2      GTTGGGGTCAAGTGAAGAGGACTCTTAGGGCTATGATGACTCAGTTCTCTTAGCCCTC
4793
1      -----
2      CAGGGCTGAACAGAACTGTTTCCCACTTATGGGTCGAGACCTCTGTTGGAGTCAGACG
4853
1      -----
2      ACCTTTCCACAGTGGTAGCCTCGGACATTGAAAATTGCAGGGGTGTTGGTTGTCGGTTG
4913
1      -----
2      GGTTTTTGTCTTGTCTGTTTGTCTTTTCAAGACAGGGTCTCTCTGTGTAGCCCTGGCTG
4973
1      -----
2      GCCTCGATTTCGGATCCACATGCCTTTCCCAAACCTGGCTTCCTTATTACTTTTTTTTTT
5033
1      -----
2      TTTTTTAAGATTTATTTACTTATTATATATAAGTACAATGTAGCTGTCTTCAGAGACACC
5093
1      -----
2      AGAAGAGGGCATCAGATCCCATACAGATGGCTGTGAGCCACCATGTGGTTGCTGGGATT
5153
1      -----

```

| | |
|------|--|
| 2 | TGAACTCAGGACCTCTTCAAGAGCAGTCAGTGCTCTTAACTTCTGAGCCCTCTCTCCAGC |
| 5213 | |
| 1 | ----- |
| 2 | CCTTTATTACTTTTAGAGATTCTTACCAGGTTGCCCTGGCTGTACTTACACTTTCTCTT |
| 5273 | |
| 1 | ----- |
| 2 | TAGCTCTGCTGAGCTTTCAATTACACAGTTCCAGACCACACCCCTGAACCCACCACTGA |
| 5333 | |
| 1 | ----- |
| 2 | AGACAACATGTCTTGCTATCTTTTTCTTTCTTTTGGGGCAGGGATGGTCTGTGTGTTG |
| 5393 | |
| 1 | ----- |
| 2 | TGTGTGTGAGAGACAGAGGCAGAGACATCCTTCCCATGTAGCCCTAACTGAGTCGGAAGT |
| 5453 | |
| 1 | ----- |
| 2 | CATTATTTAGACCAGGCTGCCCTGACTCCTGTATCTGCCTCTCCAATGGATTATGGGCAC |
| 5513 | |
| 1 | ----- |
| 2 | CTGCCACCTCACCCACTCACTTTATCCCCAGGATCCCCCTCCCTCTTCCCAGGCAGTAGG |
| 5573 | |
| 1 | ----- |
| 2 | TCTGGTGATGGAGAAGACCACTTACCATGTCCATGGCTCAAAGAAGCAGGCAGAACGTAG |
| 5633 | |
| 1 | ----- |
| 2 | GGGAAAGAGTTGCTTTACCTCAAGCCTTTGGATCCATGGGTGTTTATTCTAGTGGGGTA |
| 5693 | |
| 1 | ----- |
| 2 | ATGCCATCCTTTGAGAGCTGGGGGGTGTGGGTCAACAGAGATCGGTTGCTATGTGTGCAT |
| 5753 | |
| 1 | ----- |
| 2 | GAGGCCCGCAATTCAGTCCCTGGCTCCATGGAGAACGAACCAATCAGTTGGGTGAGGTGT |
| 5813 | |
| 1 | ----- |
| 2 | AAACCACATGTAATTTTAGCATGTCCAAACAGACACGTTAAGGACATCCTAGGGTAAAGA |
| 5873 | |
| 1 | ----- |
| 2 | AGTCTTCAGCAATAGTTTAATTGAGGCAATTATGCTCATGAATTC 5918 |

KEY

- *|| Nucleotides are identical in all sequences in the alignment
- .|| Semi-conserved substitutions are observed
- :|| Conserved substitutions are observed

Appendix 9.21 Amino Acid Sequence for *HSPA1B* *Homo sapiens* v *Rattus norvegicus*

| SeqA | Name | Len(aa) | SeqB | Name | Len(aa) | Score |
|------|------|---------|------|------|---------|-------|
| 1 | 1 | 641 | 2 | 2 | 641 | 96 |
| 1 | | | | | | |
| 60 | | | | | | |
| 2 | | | | | | |
| 60 | | | | | | |
| | | | | | | |
| | | | | | | |
| 1 | | | | | | |
| 120 | | | | | | |
| 2 | | | | | | |
| 120 | | | | | | |
| | | | | | | |
| | | | | | | |
| 1 | | | | | | |
| 180 | | | | | | |
| 2 | | | | | | |
| 180 | | | | | | |
| | | | | | | |
| | | | | | | |
| 1 | | | | | | |
| 240 | | | | | | |
| 2 | | | | | | |
| 240 | | | | | | |
| | | | | | | |
| | | | | | | |
| 1 | | | | | | |
| 300 | | | | | | |
| 2 | | | | | | |
| 300 | | | | | | |
| | | | | | | |
| | | | | | | |
| 1 | | | | | | |
| 360 | | | | | | |
| 2 | | | | | | |
| 360 | | | | | | |
| | | | | | | |
| | | | | | | |
| 1 | | | | | | |
| 420 | | | | | | |
| 2 | | | | | | |
| 420 | | | | | | |
| | | | | | | |
| | | | | | | |
| 1 | | | | | | |
| 480 | | | | | | |
| 2 | | | | | | |
| 480 | | | | | | |
| | | | | | | |
| | | | | | | |
| 1 | | | | | | |
| 540 | | | | | | |
| 2 | | | | | | |
| 540 | | | | | | |
| | | | | | | |
| | | | | | | |
| 1 | | | | | | |
| 600 | | | | | | |
| 2 | | | | | | |
| 600 | | | | | | |
| | | | | | | |
| | | | | | | |


```

1      QVCNPIIISGLYQAGAGGPGPGGFGAQQGPKGGSGSGPTIEEVD 641
2      RVCNPIIISGLYQAGAGPGAGGFGAQQAPKGGSGSGPTIEEVD 641
      :*****.*.*.*****.******

```

KEY

- | | |
|----|---|
| -* | Nucleotides are identical in all sequences in the alignment |
| -. | Semi-conserved substitutions are observed |
| -: | Conserved substitutions are observed |

| | |
|----------|-----------------------------|
| MVFILAPW | Non polar R-groups |
| GNTSYQHC | Polar R-groups |
| KR | Positively charged R-groups |
| DE | Negatively charged R-groups |

Appendix 9.22 Gene Sequence Alignment for *HSPA1L* *Homo sapiens* v *Rattus norvegicus*

CLUSTAL 2.0.12 multiple sequence alignment

```

SeqA NameLen(nt)  SeqB NameLen(nt)  Score
=====
1      1    2550      2      2    2632      78
=====

1      TTTGGAAAAAGATTACTGAGTTGGAGCCGTCTCAAATTTGCAGGGAGGGACGGGGTGGGG 60
2      -----CAGAAGAGG-----CTCAAAGTTGCCGGGAGGGGCGGGTGGGG 39
          *: ** . : **          * * * * * . * * * * * . * * * * *

1      GGTGGGGGG-ACCCCGTTGTGCAGTTTG-ATATTGAGGGAGCCCCACCTACTCGCTGG 118
2      GGTGGGGGGAACCCAGTTGCTCAGTTTGGATGTTCTGGAGCTCCCC--ACTCGTAGG 96
          * * * * * . * * * * . * * * * . * * * * . * * * * . * * * * : **

1      GGCTGCGTAATCTGGACGTTTCCAACTGAAGCGAAGGCGTCGGGAGACTAGGCCTCAGA 178
2      GGCTCAGCGACTTCAAATTTTCCAACTGGATCGAAGGCGTAGAGATTCCAGGCCTCGGA 156
          * * * . * . * . * . * * * * * * * * * * * * * * * . * * * * * . *

1      GAACCATGGCTACTGCCAAGGGAATCGCCATAGGCATCGACCTGGGCACCACTACTCCT 238
2      AAACCATGGCTGCTAATAAGGAATGGCCATAGGCATCGACCTGGGCACCACTACTCGT 216
          . * * * * * * * * . * * . * * . * * * * * * * * * * * * * * * * *

1      GTGTGGGGGTGTTCCAGCACGGCAAGGTGGAGATCATCGCCAACGACCAAGGCAACCGCA 298
2      CGGTGGGCGTGTTCCAGCACGGCAAGGTGGAGATCATCGCCAACGACCAAGGCAACCGCA 276
          * . * * * * * . * * * * * * * * * * * * * * * * * * * * * * * *

1      CCACCCCAAGCTACGTGGCCTTCACAGACACCGAGCGGCTCATTGGGGATGCGGCCAAGA 358
2      CGACCCCAAGCTACGTGGCCTTCACCGACACCGAGCGGCTCATCGGGACGCGGCCAAGA 336
          * . * * * * . * * * * * * * * * * * * * * * * * * * * * * * *

1      ACCAGGTAGCAATGAATCCCCAGAACACTGTTTTTGATGCTAAACGCTCTGATCGGCAGGA 418
2      ACCAGGTGGCCATGAATCCCCAGAACACTGTTTTTGACGCCAAACGCTCTAATTGGCAGGA 396
          * * * * * . * * . * * * * * * * * * * * * * * * * * * * * * * *

1      AATTTAATGATCCTGTTGTACAAGCAGATATGAACTTTGGCCTTTTCAAGTGATTAAATG 478
2      AGTTTAATGATCCCGTCGTGCAGTCGGATATGAAGCTGTGGCCATTCAAGTGATCAATG 456
          * . * * * * * * * * * * * * * * . * . * * * * * * * * * * * * * *

1      AAGGAGGCAAGCCCAAAGTCCTTGTGTCTTACAAAGGGGAGAATAAAGCTTTCTACCTTG 538
2      AAGCGGGCAAGCCTAAGGTGCTGGTGTCTTACAAAGGAGAGAAGAAAGCCTTCTACCCGG 516
          * * * . * * * * * * * * * * . * * * * * * * * * * * * * * * * *

1      AGGAAATCTCTTCGATGGTATTGACTAAGTTGAAGGAGACTGCTGAGGCCTTTTTGGGCC 598
2      AGGAGATCTCGTCCATGGTGTGACCAAGATGAAGGAGACTGCAGAGGCTTTTTTGGGCC 576
          * * * . * * * * * * * * * * . * * * * * * * * * * * * * * * * *

1      ACCCTGTCACCAATGCAGTGATTACCGTGCCAGCCTATTTCAATGACTCTCAACGTCAGG 658
2      ACAGTGTCACCAACGCTGTGATCACCGTGCCAGCCTATTTCAATGACTCCAGAGACAGG 636
          * . * * * * * * * * * : * * * * * * * * * * * * * * * * * * * *

1      CTAATAAGGATGCAGGTGTGATTGCTGGACTTAATGTGCTAAGAATCATCAATGAGCCCA 718
2      CCACTAAAGATGCAGGGGTCTATCGCAGGACTCAACGTGCTGCGAATCATCAATGAGCCCA 696
          * . * * * * . * * * * * * * * * * * * * * * * * * * * * * * *

1      CGGCTGCTGCCATTGCCTATGGTTTAGATAAAGGAGGTCAAGGAGAACGACATGTCCTGA 778
2      CAGCGGCGGCCATCGCCTACGGCTTGGATAAAGGAAGTCACGGAGAGCGGCACGTGCTCA 756
          * . * * * * * * * * * * * * * * . * * * * * * * * * * . * * * * *

1      TTTTGTATCTGGGTGGAGGGACATTTGATGTGTCAATTCTGACCATAGATGATGGGATTT 838

```

2 TCTTCGACCTGGGGGGCGGCACGTTTCGACGTGTCCATCCTGACGATCGACGACGGCATCT 816
* * * * *

1 TTGAGGTAAAGGCCACTGCTGGGGACACTCACCTGGGTGGGGAGGACTTTGACAAAGGC 898
2 TCGAGGTGAAGGCCACGGCGGGCGACACGCACCTGGGCGGGGAGGACTTCGACAACCGGC 876
* * * * *

1 TTTGTAGCCACTTCGTGGAGGAGTTCAAGAGGAAACACAAAAGGACATCAGCCAGAACA 958
2 TGGTGAGCCACTTCGTGGAGGAGTTCAAGAGGAAGCACAGAAGGACATCAGCCAGAACA 936
* * * * *

1 AGCGAGCCGTGAGGCGGCTGCGCACCGCCTGCGAGAGGGCCAAAGAGGACCGTGTCTGTCCA 1018
2 AGCGCGCGGTGCGGCGTCTGCGCACGGCGTGTGAGAGGGCCAAAGAGGACGCTGTCTGTCCA 996
* * * * *

1 GCACCCAGGCCAACCTAGAAATTGATTCACTTTATGAAGGCATTGACTTCTATACATCCA 1078
2 GCACCCAGGCCAACCTGGAGATCGACTCTCTGTACGAGGGCATCGACTTCTACACGTCCA 1056
* * * * *

1 TCACCAAGAGCTCGATTTGAAGAGTTGTGTGCAGACCTGTTTAGGGGTACCTTGGAGCCTG 1138
2 TCACGCGGGCGCGGTTTCGAGGAGCTGTGTGCAGACCTATTTAGAGGCACGCTTGAGCCCG 1116
* * * * *

1 TAGAAAAAGCGCTTCGGGATGCCAAGATGGATAAGGCTAAAATCCATGACATTGTTTTAG 1198
2 TGGAGAAGTCTCTCCGGGACGCCAAGATGGACAAGGCTAAAATCCATGACATTGTTCTAG 1176
* * * * *

1 TAGGGGGCTCCACCCGCATCCCCAAGGTGCAGCGGCTGCTTCAGGACTACTTCAATGGAC 1258
2 TAGGGGGCTCTACCCGCATCCCCAAGGTGCAAAAGCTGCTTCAAGACTACTTTAATGGAC 1236
* * * * *

1 GTGATCTCAACAAGAGCATCAACCGTATGAGGCCGTAGCATATGGGGCTGCGGTACAAG 1318
2 GGGATCTCAACAAGAGTATCAATCCCGATGAGGCCGTGGCGTACGGAGCTGCAGTCCAGG 1296
* * * * *

1 CAGCCATCCTGATGGGGGACAAGTCTGAGAAGGTACAGGACCTGCTGCTGCTGGACGTGG 1378
2 CAGCTATTTTAATGGGCGACAAATCTGAAAAAGTACAGGATTTGCTTTTGTGGACGTAG 1356
* * * * *

1 CTCCCCTGTCCCTGGGGCTGGAGACGGCTGGGGGCGTGATGACTGCCCTGATAAAGCGCA 1438
2 CTCCCCTGTCTCTAGGATTGGAGACAGCCGGGGGTGTGATGACTGTTCTGATCAAGCGCA 1416
* * * * *

1 ACTCCACCATCCCCACCAAGCAGACACAGATTTTCACCACCTACTCTGACAACCAACCCG 1498
2 ACTCCACCATCCCCACCAAGCAGACGCAGATCTTCAACACCTACTCGACAACCAGCCCG 1476
* * * * *

1 GGGTGCTGATCCAGGTGTATGAGGGCGAGAGGGCCATGACAAAGGACAAACACCTGCTGG 1558
2 GGGTGCTGATCCAGGTGTACGAGGGCGAGAGGGCCATGACGCGCGACAAACACCTCCTGG 1536
* * * * *

1 GGCGGTTTGACCTGACTGGAATCCCTCCAGCACCCAGGGGAGTTCCTCAGATCGAGGTGA 1618
2 GGCGCTTTGACCTGACTGGAATACCCCTGCACCTAGGGGTGTGCCCCAGATCGAGGTGA 1596
* * * * *

1 CGTTTGACATTGATGCCAATGGTATTCTCAATGTCAAGCCACGGACAAGAGCACCGGCA 1678
2 CCTTCGACATCGACGCCAACGGGATTCTCAACGTCAAGCCATGGACAAGAGCACCGGCA 1656
* * * * *

1 AGGTGAACAAGATCACCATCACCAATGACAAGGGCCGCTGAGCAAGGAGGAGATTGAGC 1738
2 AGGCCAACAAGATCACCATCACCAACGACAAGGGCCGCTTGAGCAAGGAGGAGATCGAGC 1716
* * * * *

1 GCATGGTCTGATGCTGAGAAATATAAAGCTGAAGATGAGGTCCAGAGGGAGAAAATTG 1798
2 GCATGGTGCAAGAGGCCGAGCGGTACAAGGCCGAGGACGAGGGCCAGAGGGAGAAGATCG 1776
* * * * *

1 CTGCAAGAATGCCTTAGAATCCTATGCCTTTTAACATGAAGAGTGTGTGAGTGATGAAG 1858
2 CTGCCAAAAATGCCTTAGAATCGTATGCCTTTTAACATGAAGAGCGCTGTAGGCGATGAGG 1836
* * * * *

Appendix 9.23 Amino Acid Sequence for *HSPA1L* *Homo sapiens* v *Rattus norvegicus*

| SeqA | Name | Len(aa) | SeqB | Name | Len(aa) | Score |
|------|------|---------|------|------|---------|-------|
| 1 | 1 | 641 | 2 | 2 | 641 | 94 |

=====

1
MATAKGIAGIDLGTTYSVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQ 60

2
MAANKGMAIGIDLGTTYSVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQ 60

***:

1
VAMNPQNTVFDAKRLIGRKFNDPVVQADMKLWPFQVINEGGPKVLSYKGENKAFYPEE 120

2
VAMNPQNTVFDAKRLIGRKFNDPVVSMDKLWPFQVINEAGPKVLSYKGEKKAFYPEE 120

*****:*****.*****:*****

1
ISSMVLTKLKETAEAFLGHPVTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTA 180

2
ISSMVLTKMKETAEAFLGHSVTNAVITVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTA 180

*****:*****.*****:*****

1
AAIAYGLDKGGQGERHVLIFDLGGGTFDVSIITIDGIFVKATAGDTHLGGEDFDNRLV 240

2
AAIAYGLDKGSHGERHVLIFDLGGGTFDVSIITIDGIFVKATAGDTHLGGEDFDNRLV 240

*****.*****:*****:*****

1
SHFVEEFKRKHKKDISQNKRAVRRLRTACERAKRTLSSTQANLEIDSLYEGIDFYTSIT 300

2
SHFVEEFKRKHKKDISQNKRAVRRLRTACERAKRTLSSTQANLEIDSLYEGIDFYTSIT 300

1
RARFEELCADLFRGTLEPVEKALRDAKMDKAKIHDIVLVGGSTRIPKVQRLQDYFNGRD 360

2
RARFEELCADLFRGTLEPVEKSLRDAKMDKAKIHDIVLVGGSTRIPKVQKLLQDYFNGRD 360

*****:*****:*****

1
LNKSINPDEAVAYGAAVQAAILMGDKSEKVQDLLLLDVAPLSLGLTAGGVMTALIKRNS 420

2
LNKSINPDEAVAYGAAVQAAILMGDKSEKVQDLLLLDVAPLSLGLTAGGVMTVLIKRNS 420

*****.*****

1
TIPTKQTQIFTTYSNQPGLVIQVYEGERAMTKDNNLLGRFDLTGIPPAPRGVPQIEVTF 480

2
TIPTKQTQIFTTYSNQPGLVIQVYEGERAMTRDNNLLGRFDLTGIPPAPRGVPQIEVTF 480

1
DIDANGILNVTATDKSTGKVNKITITNDKGRLSKEEIERMVLDAEKYKAEDVQREKIAA 540

2
DIDANGILNVTAMDKSTGKANKITITNDKGRLSKEEIERMVQEAERYKAEDQGQREKIAA 540

```

1      LEQMCNPIITKLYQGCGTGPACGTGYVPGRPATGPTIEEVD 641
2      LENMNCNPIITKLYQS GCTGPTCAPGYTPGRAATGPTIEEVD 641
          * *:***** * *: * ** * * *****

```

- *|| Nucleotides are identical in all sequences in the alignment
- .|| Semi-conserved substitutions are observed
- :|| Conserved substitutions are observed

Appendix 9.24 Gene Sequence Alignment for *GAPDH Homo sapiens v Rattus norvegicus*

CLUSTAL 2.0.12 multiple sequence alignment

```

SeqA NameLen(nt)  SeqB NameLen(nt)  Score
=====
1      1    1310      2      2    1307      84
=====

1      AAATTGAGCCCGCAGCCTCCCGCTTCGCTCTCTGCTCCTCC-TGTTTCGACAGTCAGCCGC
59
2      -----GGGGGCTCTCTGCTCCTCCCTGTTCTAGAGACAGCCGC
38
                                     ***** * * * *****

1      ATCTTCTTTTGCCTCGCCAGCCGAGCCACATCGCTCAGACACCATGGGGAAGGTGAAGGT
119
2      ATCTTCTTGTGCAGTGCCAGCCTCGTCTCAT-----AGACA-----AGATGGTGAAGGT
87
          ***** * * * ***** * * * * *          ***** * * *****

1      CGGAGTCAACGGATTGTCGTATTGGGCGCCTGGTCAACAGGGCTGCTTTTAACTCTGG
179
2      CGGTGTGAACGGATTGTCGTATCGGACGCCTGGTTACAGGGCTGCCTTCTCTTGTGA
147
          *** * * ***** ***** * * ***** ***** * * *

1      TAAAGTGGATATTGTTGCCATCAATGACCCCTTCATTGACCTCAACTACATGGTTTACAT
239
2      CAAAGTGGACATTGTTGCCATCAACGACCCCTTCATTGACCTCAACTACATGGTCTACAT
207
          ***** ***** ***** ***** *****

1      GTTCCAATATGATTCCACCCATGGCAAATTCATGGCACCGTCAAGGCTGAGAACGGGAA
299
2      GTTCCAGTATGACTCTACCCACGGCAAGTTCAACGGCACAGTCAAGGCTGAGAAATGGGAA
267
          ***** * * * * * * * * * * * * * * * * * * * * * * * *

1      GCTTGTCATCAATGAAATCCCATCACCATCTTCCAGGAGCGAGATCCCTCCAAAATCAA
359
2      GCTGGTCATCAACGGGAAACCCATCACCATCTTCCAGGAGCGAGATCCCGCTAACATCAA
327
          *** ***** * * * * * ***** ***** * * * * *

1      GTGGGGCGATGCTGGCGCTGAGTACGTCGTGGAGTCCACTGGCGTCTTACCACCATGGA
419
2      ATGGGGTGATGCTGGTGCTGAGTATGTCGTGGAGTCTACTGGCGTCTTACCACCATGGA
387
          ***** ***** ***** ***** ***** *****

1      GAAGGCTGGGGCTCATTTGCAAGGGGGAGCCAAAAGGGTCATCATCTCTGCCCCCTCTGC
479
2      GAAGGCTGGGGCTCACCTGAAGGGTGGGGCCAAAAGGGTCATCATCTCCGCCCTTCCGC
447
          ***** * * * * * ***** ***** * * * * *

1      TGATGCCCCCATGTTTCGTCATGGGTGTGAACCATGAGAAGTATGACAACAGCCTCAAGAT
539
2      TGATGCCCCCATGTTTGTGATGGGTGTGAACACGAGAAATATGACAACCCCTCAAGAT
507
          ***** * * * * * ***** ***** ***** *****

1      CATCAGCAATGCCTCCTGCACCAACCTGCTTAGCACCCCTGGCCAAGGTCAATCCATGA
599

```

2 TGTCAGCAATGCATCTGCACCAACCTGCTTAGCCCCCTGGCCAAGGTCATCCATGA
567

1 CAACTTTGGTATCGTGAAGGACTCATGACCACAGTCCATGCCATCACTGCCACCCAGAA
659
2 CAACTTTGGCATCGTGAAGGGCTCATGACCACAGTCCATGCCATCACTGCCACTCAGAA
627

1 GACTGTGGATGGCCCCCTCCGGGAAACTGTGGCGTGATGGCCGCGGGGCTCTCCAGAACAT
719
2 GACTGTGGATGGCCCCCTCTGGAAGCTGTGGCGTGATGGCCGTGGGGCAGCCCAGAACAT
687

1 CATCCCTGCCCTCTACTGGCGTGCCAAGGCTGTGGGCAAGGTCATCCCTGAGCTGAACGG
779
2 CATCCCTGCATCCACTGGTGCTGCCAAGGCTGTGGGCAAGGTCATCCCAGAGCTGAACGG
747

1 GAAGCTCACTGGCATGGCCTTCCGTGTCCCCACTGCCAACGTGTAGTGGTGGACCTGAC
839
2 GAAGCTCACTGGCATGGCCTTCCGTGTTCCCTACCCCCAATGTATCCGTTGTGGATCTGAC
807

1 CTGCCGTCTAGAAAACTGCCAAATATGATGACATCAAGAAGGTGGTGAAGCAGGCGTC
899
2 ATGCCGCCTGGAGAAACCTGCCAAGTATGATGACATCAAGAAGGTGGTGAAGCAGGCGGC
867

1 GGAGGGCCCCCTCAGGGCATCCTGGGCTACACTGAGCACCAGGTGGTCTCCTCTGACTT
959
2 CGAGGGCCCACTAAAGGGCATCCTGGGCTACACTGAGGACCAGGTGTCTCCTGTGACTT
927

1 CAACAGCGACACCCACTCCTCCACCTTTGACGCTGGGGCTGGCATTGCCCTCAACGACCA
1019
2 CAACAGCAAATCCCATTTCTCCACCTTTGATGCTGGGGCTGGCATTGCTCTCAATGACAA
987

1 CTTTGTCAAGCTCATTTCTGGTATGACAACGAATTTGGCTACAGCAACAGGGTGGTGA
1079
2 CTTTGTGAAGCTCATTTCTGGTATGACAATGAATATGGCTACAGCAACAGGGTGGTGA
1047

1 CCTCATGGCCCACATGGCCTCCAAGGAGTAAGAC-CCCTGGACCAACAGCCCCAGCAAGA
1138
2 CCTCATGGCCTACATGGCCTCCAAGGAGTAAGAAACCTGGACCAACCAAGCCCAGCAAGG
1107

1 GCAC-AAGAGGAAGAGAGAGACCCCTCA-CTGCTGGGGAGTCCCTGCCACACTCAGTCCCC
1196
2 ATACTGAGAGCAAGAGAGAGGCCCTCAGTTGCTGAGGAGTCCCCATCCCACTCAGCCCC
1167

1 CACCACACTGAATCTCCCCTCCTCACAGTTGCCATGTAGACCCCTTGAAGAGGGGAGGGG
1256
2 CA--ACACTGAGCATCTCC--CTCACAAATCCATCCAGACCCCAATAACAACAGGAGGGG
1223

```

1          CCTAGGGAGCCGCACCTTGTCATG--TACCATCAATAAAGTACCCTGTGC--TCAACC--
1310
2          CCTGGGGAGCCCTCCCTTCTCTCGAATACCATCAATAAAGTTCGCTGCACCCTCAAAAAA
1283
          ***  ****          ****  *  *****          *  ***  *  ****

1          -----
2          AAAAAAAAAAAAAAAAAAAAAA 1307

```

KEY

- *|| Nucleotides are identical in all sequences in the alignment
- .|| Semi-conserved substitutions are observed
- :|| Conserved substitutions are observed

Appendix 9.25 Amino Acid Sequence for *GAPDH Homo sapiens v Rattus norvegicus*

CLUSTAL 2.0.12 multiple sequence alignment

```

SeqA NameLen(aa)  SeqB NameLen(aa)  Score
=====
1      1      335      2      2      333      93
=====

1      MGKVKVGVNGFGRIGRLVTRAAFNSGKVDIVAINDPFIDLNYMVYMFQYDSTHGKFFHGTV
60
2      --MVKVG VNGFGRIGRLVTRAAFSCDKVDIVAINDPFIDLNYMVYMFQYDSTHGKFN GTV
58
          ***** . . ***** : ***

1      KAENGKLVINGNPITIFQERDPSKIKWGDAGAEYVVESTGVFTTMEKAGAH LQGGAKRVI
120
2      KAENGKLVINGK PITIFQERDPANIKWGDAGAEYVVESTGVFTTMEKAGAH LKGGAKRVI
118
          ***** : ***** : ***** : *****

1      ISAPSADAPMFVMGVNHEKYDNSLKII SNASCTTNCLAPLAKVIH DNFGIVEGLMTTVHA
180
2      ISAPSADAPMFVMGVNHEKYDNSLKIVSNASCTTNCLAPLAKVIH DNFGIVEGLMTTVHA
178
          ***** : *****

1      ITATQKTVDGPGSKLWRDGRGALQNIIPASTGAAKAVGKVIPELNGKLTGMAFRVPTANV
240
2      ITATQKTVDGPGSKLWRDGRGAAQNIIPASTGAAKAVGKVIPELNGKLTGMAFRVPTPNV
238
          ***** ***** . **

1      SVVDLTCRLEKPAKYDDIKKVVKQASEGPLKGILGYTEHQVVSDFNSDTHSSTFDAGAG
300
2      SVVDLTCRLEKPAKYDDIKKVVKQAAEGPLKGILGYTEDQVVSDFNSNSHSSTFDAGAG
298
          ***** : ***** . **** . **** : : *****

1      IALNDHFVKLISWYDNEFGYSN RVVDLMAHMASK- 334
2      IALNDNFVKLISWYDNEYGYSN RVVDLMAYMASKE 333
          ***** : ***** : ***** : *****

```

KEY

- *|| Nucleotides are identical in all sequences in the alignment
- .|| Semi-conserved substitutions are observed
- :|| Conserved substitutions are observed



MVFILAPW Non polar R-groups
GNTSYQHC Polar R-groups
KR Positively charged R-groups
DE Negatively charged R-groups

Appendix 9.26 BLAST analysis showing sequences producing significant alignments
for HSPA1A:

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident | Links |
|-----------------------------|--|-------------------------------|---------------------------------|------------------------------------|-----------------------------|-------------------------------|---|
| Transcripts | | | | | | | |
| NM_005345.5 | Homo sapiens heat shock 70kDa protein 1A (HSPA1A), mRNA | 4516 | 4516 | 100% | 0.0 | 100% | U E G M |
| NM_005346.4 | Homo sapiens heat shock 70kDa protein 1B (HSPA1B), mRNA | 3801 | 3801 | 88% | 0.0 | 99% | U E G M |
| NM_005527.3 | Homo sapiens heat shock 70kDa protein 1-like (HSPA1L), mRNA | 1620 | 1620 | 73% | 0.0 | 83% | U E G M |
| NM_002155.3 | Homo sapiens heat shock 70kDa protein 6 (HSP70B') (HSPA6), mRNA | 1580 | 1580 | 73% | 0.0 | 83% | U E G M |
| NM_021979.3 | Homo sapiens heat shock 70kDa protein 2 (HSPA2), mRNA | 1524 | 1524 | 74% | 0.0 | 82% | U E G M |
| NR_024151.1 | Homo sapiens heat shock 70kDa protein 7 (HSP70B) (HSPA7), non-coding RNA | 1474 | 1474 | 73% | 0.0 | 82% | E G M |

Appendix 9.27 BLAST analysis showing sequences producing significant alignments
for HSPA designed primers:

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident | Links |
|-----------------------------|--|-------------------------------|---------------------------------|------------------------------------|-----------------------------|-------------------------------|---|
| Transcripts | | | | | | | |
| NM_005346.4 | Homo sapiens heat shock 70kDa protein 1B (HSPA1B), mRNA | 42.1 | 82.3 | 100% | 0.027 | 100% | U E G M |
| NM_005345.5 | Homo sapiens heat shock 70kDa protein 1A (HSPA1A), mRNA | 42.1 | 82.3 | 100% | 0.027 | 100% | U E G M |
| NM_001254.3 | Homo sapiens cell division cycle 6 homolog (S. cerevisiae) (CDC6), mRNA | 34.2 | 34.2 | 52% | 6.5 | 95% | U E G M |
| NM_021979.3 | Homo sapiens heat shock 70kDa protein 2 (HSPA2), mRNA | 34.2 | 66.4 | 82% | 6.5 | 100% | U E G M |
| NR_024151.1 | Homo sapiens heat shock 70kDa protein 7 (HSP70B) (HSPA7), non-coding RNA | 34.2 | 34.2 | 42% | 6.5 | 100% | E G M |
| NM_002155.3 | Homo sapiens heat shock 70kDa protein 6 (HSP70B') (HSPA6), mRNA | 34.2 | 34.2 | 42% | 6.5 | 100% | U E G M |
| NM_022473.1 | Homo sapiens zinc finger protein 106 homolog (mouse) (ZFP106), mRNA | 32.2 | 32.2 | 40% | 26 | 100% | U E G M |
| NM_033044.3 | Homo sapiens microtubule-actin crosslinking factor 1 (MACF1), transcript variant 2, mRNA | 32.2 | 32.2 | 40% | 26 | 100% | M |
| NM_012090.4 | Homo sapiens microtubule-actin crosslinking factor 1 (MACF1), transcript variant 1, mRNA | 32.2 | 32.2 | 40% | 26 | 100% | U G M * |
| NM_006391.2 | Homo sapiens importin 7 (IPO7), mRNA | 30.2 | 30.2 | 57% | 101 | 91% | U E G M |

| Accession | Description | Max score | Total score | Query coverage | E value | Max ident | Links |
|-----------------------------|---|-------------------------------|---------------------------------|------------------------------------|-----------------------------|-------------------------------|---|
| NM_018411.4 | Homo sapiens hairless homolog (mouse) (HR), transcript variant 2, mRNA | 30.2 | 30.2 | 37% | 101 | 100% |  |
| NM_005144.4 | Homo sapiens hairless homolog (mouse) (HR), transcript variant 1, mRNA | 30.2 | 30.2 | 37% | 101 | 100% |  |